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Nociceptin and the ORL_1 receptor: analgesic mechanisms and interactions with dorsal horn neurones in rat spinal cord.

By

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ABSTRACT

There is a need to improve our understanding of the mechanisms of pain, especially neuropathic pain in order to develop new analgesic drugs based on opioids. In recent years, the cloning of the novel opioid receptor like-1 (ORL_1/NOP) receptor and studies on the effectiveness of opioids in pain models provides a basis for potential novel therapy. This thesis is based on nociceptin/orphanin FQ and its receptor ORL_1, which represent a novel peptide/receptor system pharmacologically different from classical opioid systems. Nociceptin regulates several biological functions, both at the peripheral and central levels; therefore, the ORL_1 receptor may be viewed as a novel target for drug development. However, the pharmacology of this receptor is still under study, with few molecules selectively acting on this receptor. Little is known about the physiological roles of this new opioid system.

Using an *in vivo* electrophysiology study, spinal effects of nociceptin were investigated on deep dorsal horn neurones in normal, sham operated and neuropathic rats. Nociceptin induced a greater dose-dependent inhibition in normal animals when compared with the neuropathic and sham operated animals which were the least inhibited. Additionaly to clarify the role of nociceptin and its receptor in the spinal processing of pain a non peptide antagonist and agonist of nociceptin were studied.

Another objective of this thesis was to study the interaction between nociceptin and cholecystokinin (CCK), an anti-opioid peptide. CCK enhanced the inhibitory effect of nociceptin in sham operated and neuropathic animals, whereas in normal animals CCK had the expected antiopioid action.

Furthermore, this thesis emphasizes the importance of opioid receptors located on lamina I expressing NK1 receptors in the modulation of spinal analgesia of nociceptin when compared to [D-Pen2, D-Pen5] enkephalin (DPDPE), a delta-opioid agonist.

Finally, this thesis suggests a potential therapeutic value of oxytocin in the treatment of neuropathic pain.

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I dedicate this thesis to my dad and mum for believing in me from the first day, my sisters which without them, I doubt I would have made it this far, my grandmother for all the prayers to find nice neurones and my husband for making my dreams come true.

CONTENTS

ABBREVIATIONS

β-FNA	Beta-funaltrexamine
$[Ca^{2+}]i$	Intracellular concentration of Ca^{2+}
5-HT	5-hydroxytryptamine
AMPA	α-amino-3-hydroxy-5-methyl-4 isoxazolepropionate
ASICs	Acid-sensing-ion-channels
ATP	Adenosine triphosphate
B	Bradykinin
BDNF	Brain derived neurotrophic factor
CCI	Chronic constrictive injury
CCK	Cholecystokinin
CFA	Complete Freund's adjuvant
CGRP	Calcitonin-gene related peptide
CNS	Central nervous system
COX	Cyclo-oxygenase
DAG	Diacylglycerol
DAMGO	[D-Ala^2,N-Me-Phe^4,$Gly\text{-}ol^5$]-enkephalin
DPDPE	[D-Pen^2,D-Pen^5]-enkephalin
DRG	Dorsal root ganglion
GABA	γ-aminobutyric acid
GAL	Galanin
GBP	Gabapentin
GR38032F	[1,2,3,9-tetrahydro-9-methyl-(2-methyl- 1-H-imidozol-l-yl)methyl)-4-one], ondansetron
i.p.	Intraperitoneal
i.t.	Intrathecal
ICS205-930	3-2 tropanyl-lH-indozole-3-carboxylic acid ester hydrochloride)
id.	Internal diameter
IL	Interleukins
IP3	1,4,5-inositol-triphospahte

J-113397	1-[(3R, 4 R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2 benzimidazol-2-one
LCN	Lateral cervical nucleus
L-NAME	*N*ω-nitro-L-arginine methyl ester
LRN	Lateral reticular nuclei
M3G	Morphine-3-glucuronide
m-CPBG	[1-(m-chloro-phenyl) biguanide]
mRNA	Messenger Ribonucleic Acid (mRNA)
NA	Noradrenalin
NGF	Nerve growth factor
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
nor-BNI	Norbinaltorphimine
NOs	Nitric oxide synthase
NPV	Paraventricular nucleus
NRM	Nucleus raphe magnus
NS	Nociceptive specific
NSAIDs	Non-steroidal anti-inflammatory drugs
od.	Outer diameter
ORL_1	Opioid-like receptor 1
OT	Oxytocin
PAG	Periaqueductal grey
PB	Parabrachial
PBN	Parabrachial nucleus
PK	Protein kinase
PSDC	Postsynaptic dorsal column
PSTH	Post-stimuli time histogram
PSTL	Partial sciatic tight ligation
s.c.	Subcutaneous
S.E.M	Standard error of mean
SAP	Saporin
SCT	Spinocervical tract

SG	Substantia gelatinosa
SMT	Spinomesencephalic tract
SNL	Spinal nerve ligation
SP	Substance P
SP-SAP	Substance P-saporin
SRD	Subnucleus reticular dorsalis
SRT	Spinoreticular tract
STT	Spinothalamic tract
TNF	Tumor necrosis factor
Trk	Tyrosine kinase
TTX-R	Tetrodotoxin sensitive
TTX-S	Tetrodotoxin resistant
VIP	Vasoactive intestinal peptide
VR	Vanilloid receptor
WDR	Wide dynamic range

CHAPTER 1

INTRODUCTION

Pain is defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Merskey, 1994). Pain serves an important protective function: It warns of injury that should be avoided or treated. Also pain is considered as a complex phenomenon to which an individual's response is determined by interactions between physical, psychological, cultural and social factors.

Pain can be divided into two categories, acute and chronic, which differ in their aetiology, mechanism and pathophysiology. Noxious stimulation and/or disease or abnormal functions of muscles or viscera provoke acute pain, which does not necessarily involve tissue damage. In many cases acute pain resolves within days or weeks if is treated properly. In contrast, chronic pain can persist for a long period of time, usually defined as more than 3 months, and involves tissue, nerve or visceral damage, or from dysfunction or lesions, either peripheral or central, of the nervous system. So it is not simply the duration that distinguishes acute from chronic pain but, more importantly, the inability of the body to restore its physiological function to normal homeostatic levels. Pain after tissue damage can be considered as inflammatory pain whereas nerve damage is termed neuropathic pain (Dickenson, 2001).

Whatever the type of pain, pain is a result of over stimulation of a group of specific sensory receptors called nociceptors. Their activation stimulates individual specialized neurones known as primary afferent fibres with their cell bodies located in dorsal root ganglia (DRG).
The primary afferent fibres transmit the encoded stimulus information to the dorsal horn of the spinal cord by means of a number of synapses, the first of which is within the outer dorsal horn and the output that results from spinal processing is conveyed to higher centres of the brain.

1.1 Primary afferent fibres

Both noxious and innocuous stimuli from peripheral tissues such as skin or subcutaneous tissue including muscle, bone and viscera, activates primary afferent fibres terminals.

The primary afferent fibres convey sensory information to the spinal cord, and can be classified into Aβ-, Aδ-, and C-fibres, according to their myelination, size, conduction properties and thresholds as summarised in Table 1 as well as their different termination patterns in the spinal horn of the spinal cord (Sorkin and Carlton, 1997).

Table1. Physiological properties of primary afferent fibres.

	Afferent type	Diameter (μm)	Myelination	Conduction velocity (ms^{-1})	Modality conveyed
	Aβ	6–12	Heavy	25–70	Innocuous
	Aδ	1–5	Light	10–30	Innocuous/ noxious
	C	0.2–1.5	None	<2.5	Noxious

The C-fibres, unmyelinated, slowly conducting fibres innervate nociceptors involved in the response to noxious stimuli. These nociceptors associated with C-fibres are called polymodal nociceptors since they are activated by highly-intensity mechanical, chemical or thermal stimuli (Dickenson, 2001). There are also silent or sleeping nociceptors which are unresponsive even to those intense stimuli. However, their

response is influenced by inflammatory mediators or various chemical insults which reduce their firing threshold (Dray, 1995).
The Aδ-fibres, thinly-myelinated fibres, convey both noxious and innocuous information; they respond to thermo- and mechano-stimuli less intense than those needed to exceed the C-fibre threshold.

In contrast, Aβ-fibres mediate innocuous information such as gentle touch; they have a large diameter and are heavily-myelinated. As a result of these properties Aβ-fibres have the fastest conduction velocity of the other three fibres.
The cell bodies of spinal nociceptive afferent fibres lie in the dorsal root ganglia; fibres enter the spinal cord via the dorsal root, ending in the grey matter of the dorsal horn (Fig.1).

1.1.1 Termination patterns of the afferent fibres

The large diameter primary afferent fibres enter the spinal cord through the medial division of the dorsal root. After that they descend to either the medial region of lamina I or II, or curve around the medial edge of the dorsal horn down to the ventral horn and reaching deeper laminae, lamina IV and lamina V, whilst some then ascend back up into laminae III and IV.
The fine myelinated Aδ-fibres and the unmyelinated C-fibres afferents run laterally in Lissauer's tract overlying the dorsal horn. Both Aδ- and C-fibres terminate on lamina I and II; in addition Aδ-fibres also terminate into lamina V (Sorkin and Carlton, 1997), see Fig.1.

1.2 Lamina organization and neurones of the spinal cord

The spinal cord is classically divided into the white matter (axons) and grey matter (cell bodies and their processes). Further subdivision can then be made of the grey matter into ten layers (laminae). The lamina I-VI receive sensory input and compose

the dorsal horn, lamina VII-IX contain motor neurones and compose the ventral horn, and finally lamina X is surrounded by the central canal (Sorkin and Carlton, 1997), see Fig.1. Each lamina constitutes a functionally related group of cells.

1.2.1 Lamina I

The lamina I presents the marginal layer of the dorsal horn, a thin band that covers the grey matter. Lamina I contains large marginal neuronal population that have dendrites orientated along the rostrocaudal axis, some extending into deeper lamina and most of them are projection neurones. Studies done by (Lima and Coimbra, 1986) on the basis of dendroarchitectiure and cell body shape, have identified four major neuronal types in lamina I of the spinal cord of the rat. Fusiform cells which represent 39% of lamina I neurone, multipolar (23%), flattened (13%) and pyramidal cells (25%) cells. The flattened and the pyramidal cells show the classical lateromedially elongated orientation of lamina I cells, and are also called Waldeyer cells most of which project in lateral spinocervical (SCT), spinoreticular (SRT) and spinothalamic tracts (STT) as well as to the periaqueductal grey (PAG), parabrachial nucleus (PBN) and nucleus submedius (Sorkin and Carlton, 1997). Recent studies by (Han et al., 1998) classified lamina I neurones into three types based on their morphology and receptive field properties; pyramidal and fusiform, as described by (Lima and Coimbra, 1986) and multipolar (which includes both the flattened and multipolar types of Lima and Coimbra).

Whatever the classification, lamina I neurones play a particular role in nociceptive transmission. They receive inputs from Aδ and C primary afferent fibres, including nociceptive specific (NS), wide dynamic range (WDR) and thermoreceptive cells. The NS cells respond only to intense pressure, pinch and noxious chemicals and temperatures $\geq 45^0$ C. The WDR which represent the most common spinal dorsal horn neurones, respond to gentle stimuli such as touch, brush, as well as to heat and chemicals. Finally there are also thermoreceptive cells responding to warm (35 - 43^0 C) and cooling (20 - 35^0 C) see (Wall, 1989).

1.2.2 Lamina II

Lamina II, known as the substantia gelatinosa (SG), further divided into outer layer (II_o) and inner layer (II_i), contains a densely packed concentration of small neurones and has an absence of myelinated axons (Dickenson, 2001). The SG cells are mainly stalk and islet cells. The stalk cells are located in lamina II_o, and most of their axons have ramifications in lamina I although some of them project also to deeper layers.

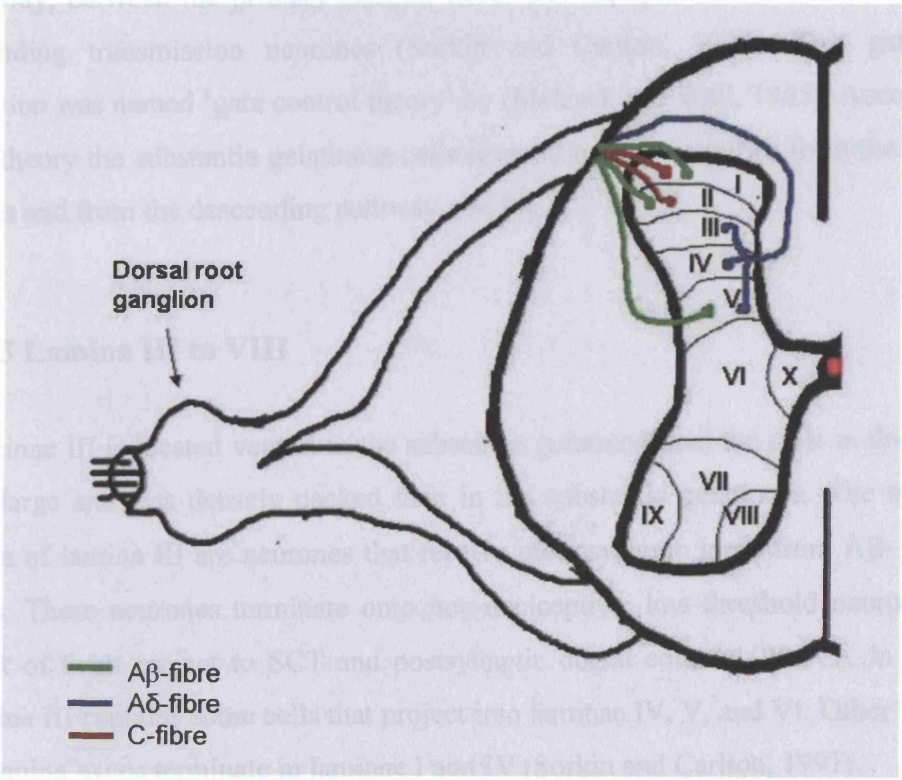

Figure 1. Morphology of the spinal cord with the laminae termination zones of the different afferent fibres.

The stalk cells predominantly relay excitatory transmission, while the islet cells are located in II_I, containing the inhibitory neurotransmitters, γ -aminobutyric acid (GABA), glycine and enkephalins in their dendrites (Dickenson, 2001). Lamina II_I neurones receive input from low-threshold mechanoreceptive primary afferent (Aδ), while those in lamina II_o respond to high-threshold primary afferent (C-fibres).

Lamina II neurones serve as interneurones, they project to lamina I and lamina V neurones where they regulate transmission at the first synapses in the nociceptive pathway, between the primary afferent fibres and the spinothalamic tract and other ascending transmission neurones (Sorkin and Carlton, 1997). This gatekeeper function was named 'gate control theory' by (Melzack and Wall, 1965). According to this theory the substantia gelatinosa cells respond to both activities from the afferent fibres and from the descending pathway, see Fig. 2.

1.2.3 Lamina III to VIII

Laminae III is located ventral to the substantia gelatinosa and the cells in this lamina are large and less densely packed than in the substantia gelatinosa. The main cell types of lamina III are neurones that receive monosynaptic input from Aβ- and Aδ-fires. These neurones terminate onto non-nociceptive, low threshold neurones, and most of them project to SCT and postsynaptic dorsal column (PSDC). In addition lamina III contains some cells that project into laminae IV, V, and VI. Other branches of lamina axons terminate in laminae I and IV (Sorkin and Carlton, 1997).

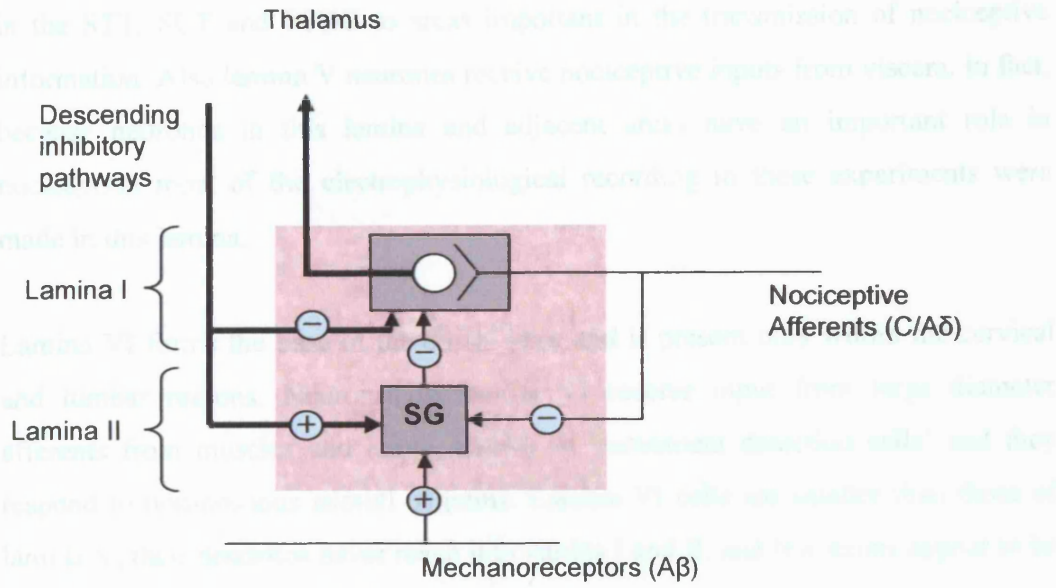

Figure 2. The gate theory proposed that neurones in the SG of the dorsal horn had their activity controlled by the balance of descending inhibition and by primary afferent fibres.

The cells in lamina IV are heterogeneous and less densely packed than lamina III. There are at least four types of lamina IV cells according to their dendritic projection patterns termination and this includes SCT and PSDC cells. The 3rd cell type has dendridic patterns similar to SCT and PSDC cells, but with axons terminating locally into the spinal cord. The fourth cell type has unknown termination axons (Sorkin and Carlton, 1997).

Lamina V presents the narrow part or neck of the dorsal horn. The cells in this lamina are more diverse than those of lamina IV and their dendrites extend vertically toward the superficial layers (Sorkin and Carlton, 1997). Lamina V neurones are primarily WDR neurones, which receive both non-noxious and noxious primary afferent input, directly from Aβ-fibre and indirectly via interneurones from Aδ- and C-fibres (Sorkin

and Carlton, 1997). The WDR cells then project to several areas of the brain such as in the STT, SCT and PSDC to areas important in the transmission of nociceptive information. Also lamina V neurones receive nociceptive inputs from viscera. In fact, because neurones in this lamina and adjacent areas have an important role in nociception most of the electrophysiological recording in these experiments were made in this lamina.

Lamina VI forms the base of the dorsal horn and is present only within the cervical and lumbar regions. Neurones in lamina VI receive input from large diameter afferents from muscles and joints, known as 'movement detection cells' and they respond to non-noxious stimuli of joints. Lamina VI cells are smaller than those of lamina V, their dendrites never reach into lamina I and II, and few axons appear to be contribute to the STT and SCT pathways (Sorkin and Carlton, 1997).

Laminae VII and VIII represent the ventral horn of the spinal cord. They are involved in pain transmission and have more complex response properties because of their polysynaptic inputs. Finally, lamina X has small cells restricted to the grey matter surrounding the central canal. Cells in this lamina have few projections in PAG, paramedial medulla and pontine reticular formation, for further discussion of other laminae see Sorkin and Carlton, (1997).

1.3 Pharmacology of the spinal cord

The primary afferent fibres, which carry nociceptive sensory information from the periphery, terminate and make their first synapse with the central nervous system (CNS) within the dorsal horn of the spinal cord. Thus this is where the peripheral input undergoes anatomical convergence and modulation by a number of neurotransmitter systems, before projection to higher brain centres via ascending tracts.

The neurotransmission of pain in the dorsal horn of the spinal cord relies on many of the same transmitters and receptors sub-serving neurotransmission in other areas of the central and peripheral nervous system. This similarity might indicate that dorsal horn pharmacology would be much the same as pharmacology at other CNS cites, (Wilcox, 1997). The next few pages will discuss the pharmacology of the spinal cord with most attention given to events that occur in the dorsal horn of the spinal cord.

The pharmacology of the spinal cord is wide and contains a diverse range of neurotransmitters and receptors, which can be excitatory or inhibitory, depending on the consequence of their receptor activation and their location. The transmission of pain can be seen as a complex mechanism involving a balance between excitatory and inhibitory systems acting at different levels of the central nervous system. All these systems are subject to plasticity and alteration during pathological conditions, so it is clear that the response to pain is not always straightforward.

The arrival of a noxious stimulus from the periphery to the dorsal horn of the spinal cord, activates the nociceptive afferent fibres (Aδ- and C-fibre), followed by the co-release of an excitatory amino acid such as glutamate, aspartate with neurokinins (predominantly substance P), calcitonin-gene related peptide (CGRP) and other peptide transmitters. At this stage, the excitatory transmission is initiated by AMPA receptor activation, which causes entry of Na^+ ions and fast depolarization of postsynaptic neurones. Only neuronal depolarization with sufficient duration and intensity is able to lead to removal of the Mg^{2+} block of the NMDA receptor channel and results in Na^+/Ca^{2+} entry. This amplified response of the nociceptive transmission

is under control by a complex inhibitory system mediated by GABA, glycine, opioid and catecholaminergic system (Hudspith and Munglani, 2003).

1.3.1 Excitatory and inhibitory transmissions

1.3.1.1 Receptors for glutamate including the N-methyl-D-aspartate (NMDA) receptor

Glutamate is the main excitatory neurotransmitter in the central nervous system, is found in most primary afferents fibres synapsing in the dorsal horn of the spinal cord, including both small and large diameter fibres. Glutamate mediates its action via the depolarisation of two distinct classes of receptors, ionotropic and metabotropic receptors (Hollmann and Heinemann, 1994). The ionotropic receptors contain cation-specific ion channels, and are further subdivided into three groups, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4 isoxazolepropionate (AMPA) and kainate receptors (Ozawa et al., 1998). On the other hand the metabotropic receptors are coupled to GTP-binding proteins (G-proteins), but their role in pain transmission is poorly understood.

AMPA and Kainate receptors are also known as non-NMDA receptors, prior to the development of selective ligands that allowed their differentiation. Recent studies have demonstrated that there are distinct receptors with different structure and functions. AMPA receptors subunits have four subunits (GluR1-GluR4) as have kainate receptors (GluR5-GluR7, KA1 and KA2) and the different assemblies of these subunits produce the different functions of native AMPA and kainate receptors (Ozawa et al., 1998). Although both kainate and AMPA receptors are widely distributed within the central nervous system, little is known about the function of kainate receptors compared to AMPA receptors.

The majority of AMPA receptors are permeable to both Na^+ and K^+, and almost impermeable to Ca^{2+}, so from this it seems that GluR1, GluR3 and GluR4 are important for Ca^{2+} permeability of the receptor complex (Ozawa et al., 1998). AMPA receptors mediate fast excitatory neurotransmission at most of the synapses in the CNS. In the dorsal horn of the spinal cord, a brief acute and more persistent stimulus from the periphery causes activation by glutamate of AMPA receptors located postsynapticaly, responsible for setting the initial baseline response to both noxious and innocuous stimuli. Subsequently, repeated and high frequency stimulation activate NMDA receptors, which is the receptor that has attracted most attention amongst the various glutamate receptors because of its complex properties and role in a number of persistent events within neuronal circuits.

Based on expression cloning techniques, it was found that the NMDA receptor has a heterometric structure composed of four subunits that include NR1 which is the fundamental subunit for the heterometric form of the receptor plus three of the four NR2 subunits (NR2A-NR2D) which determinate the functional diversity between the different NMDA receptors (Hollmann and Heinemann, 1994).
NMDA receptors in the spinal cord have gained attention particularly for their roles in nociception. As mentioned above, NMDA receptors are activated following persistent noxious stimulation, due to the unique property of this receptor compared to AMPA and kainate receptors. The NMDA receptor is blocked by physiological levels of Mg^{2+} at the resting membrane potential so that only stimuli with sufficient duration and intensity that cause repeated depolarisation of the neurone, are able to activate the receptor after the removal of the Mg^{2+} block. Other than glutamate, NMDA receptors require the binding of glycine to its site for activation and are also indirectly controlled by different peptides such as substance P which are co-released with glutamate (Dray et al., 1994) and contribute to removal of the Mg^{2+} block by actions on their receptors. Once the NMDA receptor is activated, the result is a substantially amplified postsynaptic response (Dickenson, 1995). Activation of the NMDA receptor therefore leads to the generation of spinal hypersensitivity and amplification of peripheral inputs (Besson, 1999). Furthermore, activation of the

NMDA receptor leads to entry of Ca^{2+}ions into the neurone which can then increase the activity of enzymes such as nitric oxide synthase (NOs) that generates NO. NO may be involved, in a complex way, in nociceptive events in the periphery and also within the spinal cord (Haley et al., 1992). It has been proposed that NO production appears to mediate NMDA-induced hypersensitivity and may contribute to centrally induced hyperalgesia (Kitto et al., 1992).

Glutamate and tachykinin NK1 receptors are required to generate wind-up, first described 40 years ago (Mendell and Wall, 1965). In brief, the recording of dorsal horn neurones shows that repetitive stimulation of peripheral C-fibres with a constant stimulus intensity produces a progressively increasing action potential discharge, in terms of both number and duration. This system for the amplification in the spinal cord of the nociceptive message that arrives from periphery is known as wind-up. Wind-up of dorsal horn neurones is usually evoked by C-fibres stimulation (Mendell and Wall, 1965). Also studies done by Dickenson and Sullivan, (1987a) found that blockade of NMDA receptors inhibit the generation of wind-up, underlying the importance of NMDA receptors in the mechanism of the wind-up. Although a number of different neuronal populations in the spinal cord show wind-up, WDR neurones have the most pronounced wind-up (Dickenson and Sullivan, 1987a; Herrero et al., 2000).

1.3.1.2. Substance P

Substance P (SP), an 11-amino acid peptide, together with Neurokinin A (NKA) and Neurokinin B (NKB) constitute a family of structurally related peptides called tachykinins or neurokinins. Pharmacological studies have established that substance P acts preferentially at the NK-1 receptor while the other two neurokinins (NKA and NKB) act on NK2 and NK3 receptors respectively (Brown et al., 1995). Although substance P and NKA share the same protein precursor molecule, preprotachykinin; hence is not surprising that they are found in the same sensory neurones.

All the tachykinin receptors are members of the superfamily of receptors coupled to G-proteins. The activation of these receptors leads to the stimulation of phospholipase C and thus to the generation of 1,4,5-inositol-triphospahte (IP3) and diacylglycerol (DAG) and finally to the release of Ca^{2+} from the internal stores (Krause et al., 1992). Substance P, the most abundant peptide and also the most studied of the tachykinins, is synthesised in the dorsal root ganglia, and from there migrates to the dorsal horn of the spinal cord and peripherally to the terminals of sensory neurones (Barber et al., 1979). This peptide is released from primary afferent fibres following acute noxious stimulation and there is strong evidence to suggest that substance P and its receptor play an important role in nociceptive transmission. In fact both peptide and receptor are highly distributed in many areas of the central and peripheral nervous system. In particular substance P is present in small diameter primary afferents which terminate mainly in superficial laminae I, the outer layer of lamina II and some penetrate into deeper laminae of the dorsal horn of the spinal cord (Brown et al., 1995; Todd et al., 2000). The NK1 receptor is present on certain neurones of the dorsal horn; those with cell bodies in lamina I and a population of large neurones with cell bodies in lamina III or IV and is also found on dendrites that enter the superficial laminae (Littlewood et al., 1995; Mantyh et al., 1995; Abbadie et al., 1997).
There seems to be a mismatch between the dense concentration of substance P and the amount of NK1 receptors in the different laminae, and this fact is more evident in lamina II (Littlewood et al., 1995). An explanation could be that the peptide released from the superficial laminae can diffuse over considerable distances and reach the deeper laminae at sites far from the initial synapse (Brown et al., 1995).

In the recent years substance P and its receptor gained particular attention especially after Mantyh and colleagues (1997) developed a technique for selective ablation of neurones expressing the NK1 receptor. They provided evidence that neurones in the superficial lamina expressing NK1 receptor play a critical role in the development of hyperalgesia, since this was dramatically reduced in rats with an ablation of lamina I neurones expressing the NK1 receptor. This approach attracted many laboratories

involved in pain studies and in chapter 7 of this thesis I will talk more about studies based on this technique.

Substance P is also one of the factors that initiate increased vascular permeability and protein extravasation after tissue injury in the periphery (Schaffer et al., 1998). Mantyh's group also found that after acute noxious stimulation, neurones expressing the NK1 receptor show internalization of the receptor only in their distal dendrites, while after inflammation or peripheral nerve injury the internalization sites are extended to other areas of the neurone (Abbadie et al., 1997). These observations suggest that this receptor and subsequently its peptide are important for the coding of the noxious intensity in both acute and chronic pain states.

1.3.1.3 γ-aminobutyric acid (GABA)

GABA, an inhibitory amino acid neurotransmitter, is widely distributed throughout the CNS, where it exerts its inhibitory actions. The most studied GABA receptors in the spinal cord are $GABA_A$ and $GABA_B$, both of which are found mainly pre- but also post-synaptically on nociceptive afferent fibres (Desarmenien et al., 1984). $GABA_A$ receptors are classically defined by their sensitivity to the antagonist bicuculline; these receptors are comprised of five subunits (α, β, γ, δ, ε or π) that form the walls of ligand-gated anion channels permeable to Cl^- (Bormann, 2000). Therefore their activation results in an increase in permeability to Cl^- and hyperpolarization of neurones. In addition to the GABA agonists and antagonists, $GABA_A$ receptors have also binding sites for barbiturates, benzodiazepines and various general anaesthetics (Farrant, 2001).

Unlike the $GABA_A$ receptor, $GABA_B$ receptors are G-protein-coupled receptors linked to an inhibition of Ca^{2+} influx via K^+ and Ca^{2+} channel mediated actions (Farrant, 2001). Activation of $GABA_B$ receptors causes either increases in K^+ conductance, which mediate hyperpolarization of the neurone or a decrease in Ca^{2+} influx, which causes decreases in neurotransmitter release (Hammon and Graham,

1997). Both $GABA_A$ and $GABA_B$, activation result in an inhibition of neuronal transmission. GABA-ergic terminals can also contain other inhibitory transmitters such as, glycine, galanin and neuropeptide Y (Rowan et al., 1993; Simmons et al., 1995; Todd et al., 1996).

Immunoreactive studies done by (Todd and McKenzie, 1989) showed that GABA is concentrated in interneurones of the superficial dorsal horn where mainly Aδ- and C-fibres afferents terminate. They reported that 24-33% of neurones in lamina I-III of the spinal cord contain GABA which underlines the importance of GABA-ergic systems in the modulation of nociceptive input in normal conditions and also in presence of inflammation where GABA is up-regulated (Castro-Lopes et al., 1994; Green and Dickenson, 1997). Another inhibitory system is represented by opioid receptors and will be discussed in chapter 3.

1.3.1.4 Monoamines

Serotonergic and noradrenergic neurones descend through the dorsal lateral funiculus from the brainstem to the spinal cord and terminate in the dorsal horn where they participate in the mechanisms that control impulse transmission and that significantly contribute to the modulation of pain. Electrical stimulation of brain sites such as periaqueductal gray (PAG) or the nucleus raphe magnus (NRM) produces analgesia via the local spinal release of endogenous serotonin (5-hydroxytryptamine, 5-HT) and noradrenalin (NA) see review (Furst, 1999).

The role of descending serotonergic pathways in the modulation of pain transmission is rather complicated by the multiplicity of its target 5-HT receptors and their location on the afferent fibres and/or intrerneurones, activation of which exerts both pro- and anti-nociceptive actions (Millan, 1997).

Currently, there are three major classes of 5-HT receptors: $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$ and $5\text{-}HT_4$ which are further subdivided into subtypes. These multiple receptors are

mainly present in laminae I, III and IV and therefore may play an important role in pain transmission. In this regard, particular attention has been given to the receptors 5-HT_{1A} and 5-HT_3 highly concentrated in superficial lamina I/II. The 5-HT_{1A} receptor was suggested to produce an enhanced nociception effect in the dorsal horn that may be due to inhibition of GABAergic and other interneurones (Millan, 1997).
5-HT2 receptors may mediate other inhibitory effects of serotonin by blocking the release of glutamate either by indirect actions or they may directly suppress neurotransmitter release in the dorsal horn (Obata et al., 2001). By contrast, 5-HT3 receptors directly activate a cation-permeable ion channel which triggers the opening of voltage dependent calcium channels, and they can also induce phospholipase C and further increases in intracellular concentration of calcium ($[Ca^{2+}]i$) (Millan, 1999). Thus, the $[Ca^{2+}]i$ increase will therefore cause release of neurotransmitters in the dorsal horn of the spinal cord.

Electrophysiological studies showed that under normal conditions intrathecal injection of 5-HT3 receptor agonist, m-CPBG [1-(m-chloro-phenyl) biguanide], mediates neuronal excitation while the application of 5-HT3 antagonists such as ondansetron (GR38032F) and ICS 205-930 causes a clear inhibition of nociceptive responses (Ali et al., 1996). Similar electrophysiological studies showed that during peripheral inflammation with formalin, 5-HT3 antagonist (ondansetron) decreases the response of both phases of formalin induced inflammation, suggesting that in contrast to the normal condition where there is little or no 5- HT3 receptor mediated control over nociceptive neurones in the dorsal horn, in presence of peripheral inflammation the descending serotonergic system is enhanced by acting via excitatory 5-HT3 receptors in the dorsal horn. This can then maintain or enhance the response of nociceptive spinal neurones to formalin induced peripheral inflammation (Green et al., 2000).

Moreover, studies done on knockout mice (lacking a key 5-HT3 receptor subunit) show that these animals exhibit normal acute pain responses to mechanical and thermal stimuli, however persistent pain behavior in presence of formalin or nerve

injury was significantly reduced in these animals (Zeitz et al., 2002). Although most of the studies that are mentioned above agreed the facilitatory role of 5-HT3 receptor, others have proposed paradoxical antinociceptive effects of this receptor probably by causing release of GABA, which may in turn inhibit nociceptive transmission in primary afferent fibres (Alhaider et al., 1991).

In recent years the role 5-HT3 in the descending facilitation has been further evaluated. Following depletion of lamina I expressing NK1 neurones receptor through the use of substance P conjugated with saporin which is cytotoxic (SP-SAP) (Mantyh et al., 1997), a markedly attenuated responses to pain behavior (mechanical and thermal) induced by intraplantar capsaicin injection was observed (Mantyh et al., 1997). Also electrophysiological recordings in lamina I expressing NK1 ablated animals revealed clear reduction in the characteristic response of deeper (lamina V) dorsal horn neurones such as receptive field size, mechanical and thermal evoked responses, wind-up responses as well as responses to formalin induced inflammation (Suzuki et al., 2002). Surprisingly, most of these reduced effects were reproduced by blocking the 5-HT3 in the spinal cord using ondansetron in normal animals, except the wind-up response which was not effected by ondansetron, as this spinal phenomenon is intrinsically dependent on lamina I- lamina V circuit but not influenced by descending facilitation (Dickenson et al., 2004). This data provides evidence that the 5-HT3 receptor has a key role in the descending facilitation influence from the brainstem, and underlines the importance of NK1 expressing neurones in the ascending pathway lamina I–parbrachial (PB) in the pain response (Suzuki et al., 2002).

1.4 Pathways involved in pain transmission

There are several afferent pathways from the spinal cord to the brain where the central processing of nociceptive information is initiated and the final sensation is established. These include components of the spinothalamic tract (STT) directly projecting to the thalamus, spinoreticular (SRT) and spinomesencephalic tracts (SMT) projecting to the brainstem as well as spinocervical (SCT) and postsynaptic dorsal column tracts (PSDC) (Craig and Dostrovsky, 1997).

1.4.1 Spinothalamic tract (STT)

The spinothalamic tract is the most important spinal cord pathway associated with the transmission of the information relating to pain and temperature sensation. The STT cells that terminate in the thalamus have their origins in lamina I, where 50% of STT cell are located, and the other 50% are found in lamina V and lamina VII (Craig and Dostrovsky, 1997). This was also confirmed by studies done by (Gauriau and Bernard, 2002) suggesting that the STT tract appears to be involved in nociceptive processing from deep dorsal horn neurones. As discussed previously in section 1.2 and 1.3, each lamina is characterized by different primary afferent fibres and neurones suggesting that varied information is transmitted centrally.

Briefly, STT cells include nociceptive specific neurones that respond only to noxious stimuli, thermoreceptive-specific cells excited by innocuous cooling and inhibited by warming stimuli, the WDR cells that respond to both innocuous and noxious stimuli, and other complex cells responding to both innocuous and noxious stimuli with large and bilateral somatic regions (Craig and Dostrovsky, 1997).

The properties of the spinothalamic neurones enable them to respond to a variety of stimuli and their projections mainly end on the ventrobasal thalamus making the STT tract very suitable for a role in signalling noxious stimuli (Willis, 1989).

The STT tract also projects to the lateral thalamus; including the ventral posterior nuclei (VPL, VPM); this area receives input from the superficial laminae and further projects to the somatosensory cortex, involved in the discriminative aspects of nociceptive processing (Gauriau and Bernard, 2002).

1.4.2 Spinoreticular (SRT) and spinomesencephalic tracts (SMT)

Spinoreticular and spinomesencephalic tracts are part of the spinobulbar projections, which make an important contribution to the neuronal activity underlying the motivational-affective aspect of pain, as well as somatic and autonomic motor reflexes (Willis, 1989). Overall the distribution of spinoreticular and spinomesencephalic cells is quite similar to the distribution of spinothalamic cells (Craig and Dostrovsky, 1997). The spinoreticular cells origins are primarily in the deep dorsal horn with some minor projection from laminae I and X (Sorkin and Carlton, 1997). These cells project to several reticular nuclei, such as the lateral reticular nuclei (LRN) a motor area close to the cerebellum, and the gigantocellular/paragigantocellular reticular nuclei (NGc). These projections together with the subnucleus reticular dorsalis (SRD) located in the caudal part of the medulla, make an important contribution to the motor reaction and escape behaviour induced by noxious stimuli, and also modulate the transmission of nociceptive messages in the deeper laminae (Gauriau and Bernard, 2002). In addition, descending projections from the spinoreticular area are proposed to provide inhibitory control of nociceptive input and participate in the endogenous analgesia system (Willis, 1989). However, descending controls from these areas can also be facilitatory.

The spinomesencephalic cells project mainly from laminae I and they selectively project to the lateral periaqueductal grey (PAG), while inputs from the deeper laminae terminate in both the lateral and the medial portion of the PAG (Sorkin and Carlton, 1997) and also a few cells project to the PB group (Craig and Dostrovsky, 1997). The PB area comprises several nuclei that receive dense inputs from lamina I (Craig and Dostrovsky, 1997); they respond to visceral inputs (Gauriau and Bernard,

2002). High proportions of the PB neurones project to either the amygdala or hypothalamus and are specifically excited by noxious stimuli. These projections play an important role in the motivational behaviour produced by pain, for example, emotional reactions such as anxiety, the emotional memory of pain which induces fear, and the mechanism of defence and aggression (Gauriau and Bernard, 2002).
The PB areas communicate also with the brainstem via PAG, an area involved in the passive emotional response to pain such as immobility, and hyporeactivity as well as the active emotional response of pain for example aggression, fear and other forms of hypereactivity (Gauriau and Bernard, 2002). The PAG has a central role in the mechanisms of descending inhibition and is involved in the supraspinal analgesia of opioids (Stamford, 1995), for further discussion see chapter 3.

1.4.3 Spinocervical (SCT) and Postsynaptic dorsal column tracts (PSDC)

Spinocervical tract cells are located in lamina I, III and IV where they respond to both noxious and innoxious stimuli mainly from hair follicles (Sorkin and Carlton, 1997). The lamina I terminals of this tract project to the medial lateral cervical nucleus (LCN) and the deeper cells project to the lateral portion of the LCN. Less is known of the origin of the postsynaptic dorsal column cells in rats than other species but some neurones are nociceptive (Suzuki and Dickenson, 2002).

1.5 Neuropathic pain

Neuropathic pain is initiated or caused by a primary lesion or dysfunction in the nervous system. Neuropathy can be divided into peripheral and central neuropathic pain, depending on where the lesion occurs or how the dysfunction affects the peripheral or central nervous system (Serra, 1999).

Peripheral neuropathy can result from metabolic disorders, such as diabetes mellitus (diabetic neuropathy), infections, such as herpes zoster (post-herpetic neuralgia) and human immunodeficiency virus, nerve compression due to tumours, as well as neuroma formation arising from amputation or nerve transection (Garry, 1997). Central mediated neuropathic pain can result from disorders of the spinal cord such as multiple sclerosis and trauma or tumours in different region of the spinal cord and brain. This pain state differs from acute pain because the therapy provides only transient pain relief and does not resolve the underlying pathological process, and in fact, chronic pain will continue when the treatment stops (Loeser, 1999).

Peripheral nerve disorders are the major class of neuropathic pain encountered in patients. There are several symptoms of neuropathic pain, the most studied ones are associated with spontaneous pain, hyperalgesia and allodynia; which seriously compromise the quality of life of the patient.

Spontaneous pain, is a pain perception arising from the innervation area of the injured nerve and can occur in the absence of an external stimulus. This stimulus independent pain can be continuous (ongoing) or paroxysmal (episodic) pain and is often described by the patient as having 'tingling, crawling, cramping, burning or stabbing' components (Bennett, 1994). This type of pain appears to be common in patients with central and peripheral nerve damage.

In contrast, neuropathic pain is also characterised by the presence of stimulus-evoked pain and has two key features: allodynia and hyperalgesia. Allodynia is the sensation

of pain evoked by a normally non-noxious stimulus such as gentle touch and brushing. It can be produced by the activation of low threshold myelinated Aβ-fibres. A theory was proposed by Woolf et al., (1992), when they observed that after nerve injury there was a significant sprouting of the large Aβ-fibres into the superficial lamina of the dorsal horn of the spinal cord, area that normally receive nociceptive input. This was taken as suggesting that the sprouting could lead formation of anomalous synapses with cells important for the transmission of nociceptive information (Ossipov et al., 1997). Recent work casts doubt on this theory since the tracer used may no longer be selective for large fibres after nerve injury and now is taken up by damaged fine fibres (Hughes et al., 2003).

Allodynia can be further defined in terms of the evoking stimulus, such that 'dynamic' or tactile allodynia arises from a moving stimulus in contrast to the 'static', 'pressure' and 'punctate', allodynias, produced by large or fine-tipped probes, respectively. Tactile allodynia sensation is one of the major debilitating pain symptoms among patients with neuropathic pain since even gentle contact with clothes can evoke intense pain sensations. In combination with the mechanical allodynia there may also be warm- and cooling-allodynia which are perceptions of pain produced by warm and cold stimuli respectively (Bennett, 1994).

Another stimulus-evoked pain is represented by hyperalgesia, which is described as an enhanced pain perception, such that there is an abnormal increased pain response to a normally noxious stimulus. As with the allodynia, the hyperalgesia can be further classified as mechanical, heat and cold hyperalgesia. Although allodynia and hyperalgesia are conceptually different, they are often confused or misinterpreted. The main difference between the allodynia and hyperalgesia is represented thus; while allodynia represents a qualitative change, hyperalgesia is more a quantitative change in the perception of pain.

Paradoxically, despite the sensory deficit that results from the sensory loss in neuropathic states, pain can on occasions, be evoked from that same area.

Hyperpathia is the term given to that condition: a painful syndrome, characterized by increased reaction to a stimulus where the pain continues after the painful stimuli causing the pain stops. Hyperpathia can be evoked by both normal innocuous and normal noxious stimuli.

This classification of aetiology and symptoms presents part of the framework of neuropathic pain which is a complex state and still, the relation between the symptoms and underlying mechanisms is not fully understood. Several mechanisms may be at work in an individual patient, and the same mechanisms may not be present in all patients who share a specific neuropathic pain state. Furthermore one mechanism could be responsible for many different symptoms and this may change with time. This variability suggests that neuropathic pain can result through diverse mechanisms and consequently the treatment of neuropathic pain will be complex. In order to further clarify our understanding in the mechanisms of neuropathic pain it has been necessary to develop valid animal models of this clinical state.

1.5.1 Animal models of neuropathic pain

Basic scientific research into the causes and possible treatment of neuropathic pain has accelerated with the development of animal models that reflect some of the elements of clinical pain syndrome.

Earlier models of neuropathic pain involved a complete lesion or crush or nerve section to mimic the state seen after amputation, but more recently with the recognition that most neuropathic pain patients have a partial nerve injury, models which preserve some of the sensory information passing into the spinal cord become more common. The most popular of these are the mononeuropathy models which are the chronic constrictive injury (CCI) model (Bennett and Xie, 1988), partial sciatic tight ligation (PSTL) model (Seltzer et al., 1990) and the selective spinal nerve ligation (SNL) model (Kim and Chung, 1992), which are represented in Fig.3.

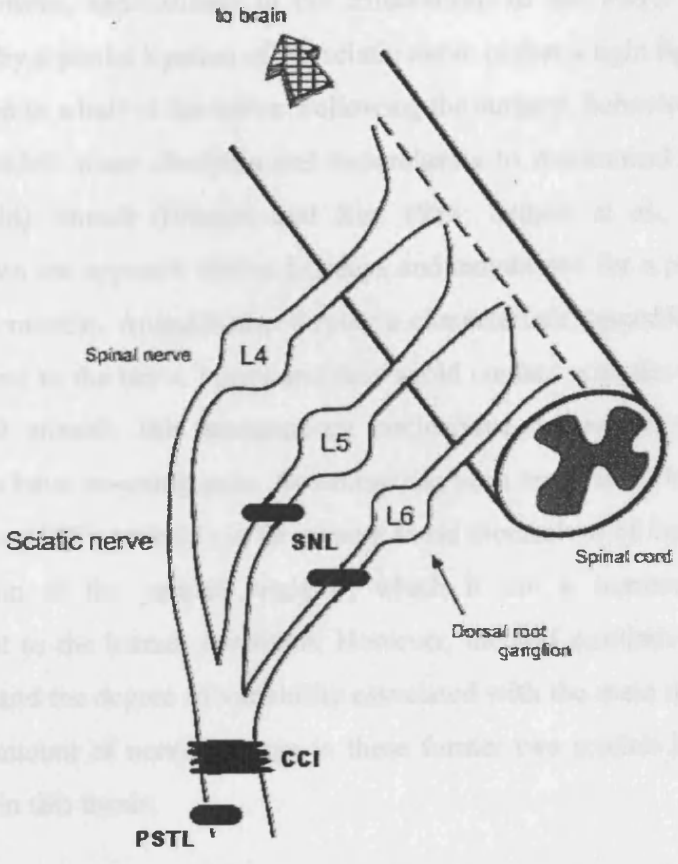

Figure 3. Animal models of neuropathy. PSTL (partial sciatic tight ligation) involves tight ligation of 1/3 to 1/2 of the sciatic nerve; CCI (chronic constrictive injury) consist of loose ligature around the sciatic nerve. Finally the SLN (spinal nerve ligation) model used in this thesis involves tight ligation of L5 and L6 spinal nerves. Figure modified from (Decosterd and Woolf, 2000).

The CCI model involves placement of four loose chromic catgut ligatures around the sciatic nerve, approximate to the trifurcation. In the PSTL model, neuropathy is caused by a partial ligation of the sciatic nerve in that a tight ligature is placed around one third to a half of the nerve. Following the surgery, behavioural studies reveal that both models show allodynia and hyperalgesia to mechanical and thermal (both hot and cold) stimuli (Bennett and Xie, 1988; Seltzer et al., 1990). These evoked responses are apparent within 1-2 days and maintained for a post-operative period of up to 3 months. Animals also display a characteristic 'guarding' posture on the side ipsilateral to the nerve injury and they avoid contact with the floor in the absence of external stimuli; this spontaneous nociceptive behaviour may indicate that the animals have on-going pain. Autotomy has been reported in the CCI model (Bennett and Xie, 1988), probably in an attempt to rid themselves of the pain or the unpleasant sensation of the painful hindpaw, which is not a common clinical observation relevant to the human condition. However, the self mutilation observed in the CCI model and the degree of variability associated with the main difficulty of creating the same amount of nerve damage in these former two models lead to the use of SNL model in this thesis.

The SNL presents an alternative approach to the CCI and PSTL models, and was developed by Chung and colleagues using partial ligation of the sciatic nerve by tight ligation of the L5 and L6 of the spinal segmental nerve, leaving the L4 component of the sciatic nerve intact in order to avoid motor deficits, see section 2.1. in the methods for the surgical description of this model. In this model rats develop long lasting hyperalgesia to noxious mechanical and thermal stimuli as well as mechanical and cold allodynia which persist for up to 2 months (Kim and Chung, 1992). However, Kim and Chung observed that the ipsilateral hind paw became extremely sensitive by 2 weeks after the surgery, and can be seen in Fig.1 of the methods section where the response to the lowest von Frey show further increases of foot withdrawals in day 14 of the postoperative period, which indicates strong presence of mechanical allodynia around that period. In fact the electrophysiological studies of these animals are made after day 14.

Using this model rather than the CCI and PSTL, since the same spinal nerves (L5/L6) in each animal are tightly ligated in the SNL model, so limiting the variability in the type and number of injured fibres between the experiments (Kim and Chung, 1992). An unique property of this model is presented by the fact that the location of the injured fibres are in completely separate segments from the uninjured fibres, since the dorsal root ganglia (DRG) which contains the injured nerves is separated from the adjacent DRG containing intact neurones (Kim and Chung, 1992). The situation is opposite in the CCI and PSTL, where the injured and the intact fibres are mixed.

The only variability in the SNL model is normal biological variability where differences between individual rats may exist in the proportion of the sciatic nerve contributed to by the 3 spinal nerve segments.

The behavioural consequences of these nerve injuries emphasizes that these animal models present predictable changes in the behaviour that are close parallels to the effects of injuries in humans. Investigating the altered functions-anatomical, electrophysiological and neurochemical in the neuropathic pain is important to provide more understanding of mechanisms and insights into the corresponding pain state in humans.

1.5.2 Mechanisms of neuropathic pain

In normal conditions as described above, the mechanisms by which a noxious or nociceptive stimulus is evoked, transmitted and finally perceived are complex. At each of these functional levels there is a balance between excitatory and inhibitory systems. In neuropathic pain this process becomes more complicated due to both the interruption of the normal connections of the system and the dynamic compensatory responses that occur throughout the nervous system. There are changes related to the tissue damage which cause excitability changes in the periphery and central nervous system that establish healing of the nerve injury and are reversible responses. Other changes are more permanent and persist despite resolution of the original neuropathic

insult. This latter group is likely to correlate closely with neuronal plasticity that may be responsible for the chronic neuropathic pain syndrome. Some of these changes associated with nerve injury will be discussed below.

1.5.3 Anatomical changes within peripheral nerves

The sensory neuronal pathway starts within the primary afferent neurones that are characterized by a cell body located within the dorsal root ganglia (DRG) and a stem process that bifurcates into a central process that enters the dorsal horn of the spinal cord and peripheral processes that target tissue. The afferent fibres of this nerve, according to their degree of myelination and other properties discussed in section 1.1 are classified into A- and C-fibres. In normal condition the myelinated fibres, myelin is important to facilitate the propagation and the conduction of impulses and their ongoing transmission along the axon. Also present are supporting Schwann cells with a primary function to myelinate and promote rapid nerve impulse transmission located along the axon of the peripheral nerves (Gillespie et al., 2000).

Following peripheral nerve injury, there are changes in the anatomy of the peripheral nerves that may explain the generation of neuropathic pain. Peripheral nerve damage usually results in the formation of a 'neuroma', a swollen end, due to continued axonal transport. In a state of nerve injury, in the same way as inflammation can induce infiltration of macrophages and immunocompetent cells (further discussion see section 1.6) these cells could damage nerves and DRG. This damage is thought to be mediated by the phagocytotic action of macrophages that invade the site of nerve injury following nerve injury. All these insults, together with a disruption to Schwann cell distribution and density that occurs along the damaged axon produces areas of demyelination, which causes disruption in the axonal transport essential for the maintenance and repair of the axonal process yet also leading to an alteration of conduction or excitability of the injured nerve (Garry, 1997). Following nerve injury there is a substantial decrease in the number of sensory axons distal to the site of damage accompanied by sensory neuronal cell death in the DRG. Is not surprising

that the major loss involves the largely myelinated Aβ- and Aδ-fibres, which reach up to 89% and 55% of fibres lost, respectively, with little reduction in C-fibre numbers (Garry, 1997).

Furthermore, following injury the denervated peripheral tissue does not passively wait for reinnervation, but rather, it actively responds to the lack of electrical and chemical communication by inducing direct regenerative efforts and sprouting of the axon to attempt to regain their appropriate connection to the central nervous system (Colburn and Munglani, 2003). The regeneration of injured peripheral nerves appears to be dependent on the presence of regeneration-promoting substances such as nerve growth factor (NGF) and apolipoprotein E in the outgrowth region (Garry, 1997). These substances are known to promote the development of the nervous system and their role in the myelination of the newly grown axon, respectively. Regeneration of the nerve and consequently the recovery of the target organ function are not always successful; it depends on fibre type of nerve fibre and the severity of nerve injury. It appears that large, myelinated Aβ-fibres are least likely to regenerate, in comparison to C-fibres (Navarro et al., 1994).

In addition to these attempts to provide direction for the regenerating axon, however, uninjured sensory axons from the neighbouring nerves expand into the area previously innervated by the lesioned nerve. This phenomenon is termed collateral sprouting and leads to altered sensory properties that includes expanded receptor fields, sensitized stimuli transduction, and changes in central synaptic connectivity (Colburn and Munglani, 2003). All these altered sensory properties suggest that the collateral sprouting may be implicated in the development of hyperalgesia. An example can be presented by experiments done in the model of CCI, showing a marked hyperalgesia, where the hyperalgesia was prevented by saphenous nerve section to prevent collateral sprouting, one week after nerve injury (Ro and Jacobs, 1993). A similar case may also occur in the partial denervation where the collateral sprouting can form connection with the damaged nerve. So it appears that following

injury aberrant connections can cause both peripheral and central plasticity that contributes to the development of neuropathic pain.

Another important consequence following nerve injury is abnormal nociceptor sensitization, where the primary afferent fibres respond with increased sensitivity to various stimuli (mechanical, thermal and chemical) due to the local release of inflammatory mediatory such as bradykinin, histamine, prostaglandins and leukotrienes (Garry, 1997). This action of mediators may contribute to the maintenance and the development of hyperalgesia and allodynia following nerve injury.

1.5.3.1 Ephaptic transmission

In normal, uninjured physiological conditions each primary afferent neurone transmits impulses through independent sensory channels in the peripheral nervous system until convergence occurs in the dorsal horn. This is made possible by the myelination of A-fibres, and the presence of non-neuronal glia cells (Schwann cell) processes that separate them from unmyelinated C-fibres.
In contrast, following nerve injury the insulation of these processes can be disrupted as discussed previously and as consequence the impulse can be transmitted to the neighbouring fibres, leading to a phenomenon called ephaptic transmission or cross talk (Garry, 1997). This phenomenon maybe contributes to the expanding of the receptor field and so the central nervous system will not receive an accurate coding of information. All these may therefore contribute to the abnormal sensations related to neuropathic pain and explain how innocuous stimulation can result in perception as a noxious stimulation or vice verse as part of the abnormal response to the stimulus.

1.5.3.2 Ectopic activity

Action potentials can travel in both directions in sensory axons. With nerve injury, ectopic discharge can result in antidromic action potentials leading to release of neuropeptides. These neuropeptides, such as substance P and CGRP, can lead to the sensitization of the peripheral sensory terminal of injured and uninjured fibres (Woolf and Mannion, 1999). As a result, sensitization of primary afferent nociceptors (Aδ- and C-fibre) is characterized by ongoing discharges, a lowered threshold for activation, and an increased response to a given stimulus.

Following nerve injury, it has been demonstrated that there is reorganization of the nature and levels of expression of the various channels, (see section 1.5.4). Sodium channels are one of the most important channels, indispensable for the conduction of neural impulses and changes in the expression of these channels are thought to contribute to the generation of the ectopic activity that can occur in the damaged primary afferent fibres, their DRG and also areas of demyelination along the axon of the injured nerves,see review (Teng, 2003).

Ectopic activity that occurs within the DRG is slow and irregular compared to the rhythm and high frequency of the ectopic activity in most injured afferent site. These ectopic discharges would then enter the spinal cord and sensitize spinal dorsal horn neurones (Yoon et al., 1996). The ectopic afferent activity is largely responsible for the development of hypersensitivity of spinal cord dorsal horn neurones and the hyperalgesia and allodynia associated with neuropathic pain in rats (Yoon et al., 1996). Clinical studies also provide strong evidence that the ectopic discharges from afferent nerves are a source of ongoing spontaneous pain, and this abnormal afferent activity dynamically maintains a state of central hypersensitivity that underlies evoked-pain syndromes such as allodynia and hyperalgesia in patients with painful neuropathies (Gracely et al., 1992; Campero et al., 1998). Thus, drugs capable of suppressing the ectopic afferent activity may provide an effective therapy for the

treatment of neuropathic pain. In this regard, the analgesic effect of anticonvulsants may be mediated partly by their action on ectopic afferent discharges (Pan et al., 1999). Mechanisms underlying the generation of ectopic discharges from injured afferents are complex and likely to involve the interaction between endogenous chemicals and ion channels.

1.5.4 Alteration of ion channels in nerve injury

Ion channels such as Na^+, Ca^{2+} and K^+ regulate neuronal excitability and are involved in the initiation and propagation of action potentials along nerve axons.
Since impulse initiation at normal localization is dependent on Na^+ channels, several studies have been done to understand changes that occur in the expression of these channels in DRG neurones following injury. In nerve injury there is both a triggered hyperexcitability and the ectopic initiation of impulses in primary afferent fibres.

In the sensory neurones two classes of functionally distinct Na^+ channels are present, which are classified as tetrodotoxin sensitive (TTX-S) and TTX resistant (TTX-R) Na^+ channels. The TTX-S Na^+ channels are expressed in large and small diameter sensory neurones and are inactivated and sensitive to submicromolar concentration of TTX. While the TTX-R Na^+ channels including the Nav1.8 and Nav1.9, are insensitive to submicromolar concentration of TTX and both channels are slow activated channels (Zhang et al., 2004). The small diameter nociceptive fibres express both TTX-S and TTX-R Na^+ channels, this preferential expression of TTX-R in the nociceptive fibres suggesting the importance of these channels in the pharmacological treatment of pain (Waxman et al., 1999).

Previous studies have demonstrated that changes in Na^+ channel expression at both DRG and the site of nerve injury occur after nerve injury. In particular a down-regulation of both TTX-R channels (Nav 1.8 and Nav1.9) and a TTX-S Na^+ channel (Nav 1.7) as well as up-regulation of another TTX-S Na channel (Nav 1.3) were shown in DRG neurones, for further discussion see Zhang et al., (2004).

On the other hand, immunolocalization studies showed that the level of Na^+ channels increases at the site of nerve injury (Devor et al., 1993) and this was due to an accumulation of Nav 1.8 and Nav 1.9 at the site of nerve injury (Coward et al., 2000).

As a result, changes in the expression of Na^+ channels are thought to contribute to spontaneous activities, hypothesized to be one of the mechanisms underlying neuropathic pain (Dib-Hajj et al., 1999; Waxman et al., 1999; Zhang et al., 2004). Since low doses of TTX block the TTX-S Na^+ channels, which in turn inhibits ectopic firing originating from neuromas, DRG and dorsal horn neurones (Omana-Zapata et al., 1997) and reduces neuropathic behaviors such as allodynia (Lyu *et al.,* 2000), it therefore appears that TTX-R and TTX-S Na^+ channels may play differential roles in pain transmission with a significant contribution of TTX-R Na^+ channels in uninjured neurones and TTX-S Na^+ channels in injured neurones (Zhang et al., 2004).

Furthermore, Na^+ channel blocker drugs including local anaesthetics and anticonvulsants that inhibit the opening of the Na^+ channels, when applied directly to the nerve or administrated systemically reduce ectopic neuronal discharge, further discussion of the pharmacological treatment of neuropathic pain see section 1.5.7.

Sodium channels are not the only channels that are involved with neuropathic pain. Calcium channels have been shown to affect the development of hyperalgesia and allodynia. Several studies have demonstrated that hyperalgesia and allodynia can be reduced with the application of specific antagonists for neuronal N-type calcium channels, further discussion see section 1.5.7.

1.5.5 Neurochemical changes in nerve injury

In nerve injury there are complex mechanisms since the sensory neurones and the non-neuronal cells associated with them are highly interdependent. They form an ecosystem, where a change in one of them affects many other systems. In the previous section I described changes related to anatomy, sensory property transmissions and some channels, important for the mechanisms of neuropathic pain. In addition to these changes it is believed that the sensory neurones in the DRG respond to the injury by altering the synthesis of various proteins in an effort to support and regenerate the injured neurones (Garry, 1997). More specifically, nerve injury triggers changes in gene expression in the DRG, where in some cases increases and in others, decreases in proteins are seen.

1.5.5.1 Neurochemical up-regulation

The presence of nerve injury cause an increase in the number of cells immunoreactive for vasoactive intestinal peptide (VIP) in the primary sensory neurones of the dorsal horn of the spinal cord and their DRG (Shehab and Atkinson, 1986). VIP is highly expressed in certain sacral afferents, but the fact that VIP gene expression is induced in the lumbar ganglia following injury suggest that VIP may be playing a role in control of neuronal degeneration and elicit a survival-enhancing effect as seen on neurones *in vitro* and *in vivo* (Hokfelt et al., 1994). Further, it is present in both A- and C-fibre where it acts as a facilitatory neurotransmitter in the dorsal horn, and this role may be accentuated subsequent to nerve injury. Thus, VIP assumes a major, excitatory role in nociceptive transmission in neurones in the dorsal horn following its induction by peripheral afferent fibres injury (Shehab and Atkinson, 1986); (Hokfelt et al., 1994).

Similar to VIP, galanin is also upregulated following nerve injury. In intact animals, the neuropeptide galanin is expressed in a small number of DRG neurones, preferentially in the small diameter cells and it is characterized by both facilitatory

and inhibitory effects (Wiesenfeld-Hallin et al., 1992). This biphasic effect of galanin, might be due to the fact that there are several galanin receptors involved. In fact there are three cloned galanin receptors, GalR1, 2 and 3 (Branchek et al., 2000). The galanin receptors in spinal cord and DRG are mainly represented by GalR1 and GalR2. In normal animals low levels of galanin produce a pro-nociceptive response but the peptide inhibits at higher doses (Wiesenfeld-Hallin et al., 1992). Thus, after nerve injury where galanin receptor is upregulated, spinal administration of galanin causes predominantly inhibitory effects in the dorsal horn (Wiesenfeld-Hallin et al., 1992; Hokfelt et al., 1994). Furthermore, experiments carried in rats with intact nerves, galanin antagonizes the facilitatory effect of substance P and CGRP. This condition is altered following injury; galanin in this case has no effect on exogenous substance P, but counteracts the facilitatory effect of VIP (Xu et al., 1990).

Another neuropeptide recently shown to be associated with sensory neurones is neuropeptide Y, which is up-regulated after nerve injury and mostly is present in the large myelinated Aβ-fibres (Wakisaka et al., 1991). The intrathecal injection of neuropeptide Y induced a similar biphasic effect to galanin, with a facilitatory effect at lower doses and inhibitory effect at higher does (Xu et al., 1999). This biphasic effect may be related to several subtypes of neuropeptide Y receptor. In fact, anatomy and functional data suggest that neuropeptide Y receptors are heterogenous and can be classified into six subtype receptors Y1-Y6, where Y2 are mainly located in DRG neurones and Y1 in some dorsal horn interneurones (Xu et al., 1999). Both peptide and receptors in the DRG and in the spinal cord undergo marked plasticity after nerve injury. Nerve injury induces an up-regulation of neuropeptide Y which increases both its excitatory and inhibitory effects (Xu et al., 1994).

Furthermore, nerve damage causes also alterations of neuromodulators in primary afferent fibres, such as nitric oxide (NO). Several studies suggested that spinal NO, a neuronal messenger, synthesised from L-arginine by nitric oxide synthase (NOs), which is found throughout the CNS, is involved in the mechanism of pain induction. In this regard, epidural injection of L-arginine produces a slowly developing thermal

hyperalgesia in rats (Masue et al., 1999). This facilitatory effect of NO can be explained by the fact that spinal NO release is evoked by NMDA receptor activation suggesting that NO production and its extracellular movement may be linked in the pathways that follow NMDA receptor activation (Bogdanov and Wurtman, 1997) see also section 1.3.1.1. Also, nerve injury induced either by spinal nerve ligation (Luo et al., 1999) or sciatic nerve transection (Verge et al., 1992) causes marked increases in the expression of messenger RNA level of neuronal NOs in the DRG. Since, a nonspecific NOs inhibitor, *N*ω-nitro-L-arginine methyl ester (L-NAME), attenuates hyperalgesia and allodynia following nerve injury (Yoon et al., 1998), suggesting that spinal NO production may contribute to the control of pain behaviours following nerve injury. On the other hand administration of NOs inhibitors failed to decrease the background activity of dorsal horn neurones and to reverse the allodynia in normal and nerve injured rats respectively (Luo et al., 1999; Hoheisel et al., 2000). However, although the level of NO is upregulated after the nerve injury, the correlation between neuronal NOs expression and the development and/or maintenance of allodynia is not clear.

Finally, following nerve injury there is an increase level of cholecystokinin (CCK). This peptide is widely distributed in several areas of CNS important in the modulation of nociceptive transmission. Although CCK does not alter baseline pain thresholds (Xu and Wiesenfeld-Hallin, 1997), it is believed that CCK is partly responsible for the reduced opioid actions in neuropathic pain and this will be discussed in chapter 6.

1.5.5.2 Neurochemical down-regulation

In contrast to the increased expression of neuropeptide Y, VIP, galanin and CCK discussed above, some other peptides decrease in the DRG following nerve injury. Studies have reported that following nerve injury there is down-regulation of peptides such as CGRP and substance P (Cameron et al., 1991).

CGRP and substance P, as mentioned in section 1.3, both facilitate transmission in the dorsal horn of the spinal cord. Since noxious peripheral stimulation evokes a release of these peptides in the dorsal horn of the spinal cord (Morton and Hutchison, 1989) it has been established that both CGRP and substance P are colocalized in most primary sensory neurones, although CGRP is present in 50 % of neurones of all sizes while substance P is present in 20% of neurones of small size (Nothias et al., 1993; Hokfelt et al., 1994). These observations together with the finding that intrathecal administration of CGRP elicits hyperalgesia (Yu et al., 1994), have led to the suggestion that this peptide may have a role in nociceptive transmission and modulation. CGRP exerts its pronociceptive effect via two receptor types, $CGRP_1$ and $CGRP_2$, both of which are positively coupled to adenyl cyclase, however it is not clear what the respective role of these receptors is in the mediating the action of CGRP (Millan, 1999).

In addition to the direct role of CGRP in the spinal cord, CGRP potentiates the effect of substance P by inhibiting substance P endopeptidase, an enzyme that catalyzes the substance P within the amino acid chain (Hokfelt et al., 1994). In nerve injury it seems that the role of pain transmitting mediators such as substance P and maybe also that of CGRP is taken over by VIP (Wiesenfeld-Hallin et al., 1990). This was suggested by studies done in normal animals which showed that a substance P antagonist attenuated the facilitatory effect of substance P, while in nerve injury the antagonist had no effect and surprisingly a VIP antagonist which had no effect under normal conditions antagonized the effect of substance P following injury (Wiesenfeld-Hallin et al., 1990).

As opioid receptors are down regulated in presence of injury this may explain the reduced effect of this class of drugs in nerve injury, and further discussion of the opioid system is in chapter 3.

These changes in the neuropeptide expression underline the complexity of the neuropathic mechanisms and subsequently the treatment of neuropathic pain. The overall effect is a state of spinal cord disinibition and increased receptivity to incoming stimuli.

1.5.6 Sympathetic system

Under physiological conditions, the activity of the primary afferent fibres nociceptors is unaffected by sympathetic outflow, due to the lack of or low catecholamine sensitivity (Janig et al., 1996). However under pathological conditions such as nerve injury or inflammation, an interaction between afferent fibres and sympathetic system seems to be established. To elucidate the role of sympathetic influence in the pathological condition, several studies have been conducted in both animals and humans.

After nerve lesion, sympathetic postganglionic fibres that normally innervate blood vessels within the DRG sprout to form basket-like terminals around primary afferent soma that project into the lesioned nerve (McLachlan et al., 1993). These unusual connections provide a possible origin for abnormal discharge following peripheral nerve injury. Furthermore it has been observed that sympathetic stimulation can activate the lesioned nerve repetitively, suggesting that the sprouts of sympathetic axons from physical contact with the DRG of primary afferent fibres might trigger release of some substances in the vicinity of the peripheral afferent fibres and within the DRGs (McLachlan et al., 1993; Chung et al., 1997).

In addition, studies done on SNL animals with stable mechanical allodynia and thermal hyperalgesia showed that surgical sympathectomy performed prior to the SNL reverses the sensitivity of the innocuous mechanical and thermal stimuli developed in the injured paw, suggesting a contribution of sympathetic innervation in the development of mechanical allodynia and thermal hyperalgesia produced by peripheral nerve injury (Kim et al., 1993). Also after chemical sympathectomy sympathetic blocking agents such as phentolamine and guanethidine, rather than surgical sympathectomy caused a reduction in the mechanical allodynia and thermal hyperalgesia produced by peripheral nerve injury (Kim et al., 1993). Moreover, similar results after surgical and chemical sympathectomy have been reported in PSTL and CCI models (Neil et al., 1991; Shir and Seltzer, 1991).

Overall the present studies show the importance of a sympathetic maintenance in these models of neuropathic pain, however reversal of neuropathic pain behaviours has not been successful in L5 spinal nerve ligated rats (Ringkamp et al., 1999). An important point in these experiments is the involvement of adrenoceptors in sympathetic- afferent coupling. Although the cellular mechanism underlying this interaction is not clear, an obvious explanation could be the increased sensitivity of adrenoceptors as a consequence of nerve lesion or infection (Baron, 2004). In normal conditions although the adrenoceptors are present on primary afferent fibres, they may not be functional. It is believed that the sympathetic-afferent coupling is largely mediated by alpha 2-adenoreceptors (Sato and Perl, 1991), and stimulation of this receptor may mediate increases in the spontaneous activity in nerve injury. Furthermore up-regulation of the mRNA for alpha 2-adenoreceptors in the DRG neurones has been confirmed after nerve lesion (Shi et al., 2000).

However, there is a discrepancy in the literature on the role of the sympathetic influence in nerve injury, that may well be explained by differences in the animal models used since types of peripheral nerve damage and animal strains may provide sources of variability in the generation and evolution of sympathetic sprouting. Alternatively, as seen in the classical review (Bonica, 1990) blockade of sympathetic innervations to the effected area is effective in alleviation of the pain symptoms only in certain patients. Thus sympathetic maintained pain is not seen in all patients with nerve injury. In addition clinical studies show that human peripheral painful neuropathy varies considerably in the development of adrenergic sensitivity, despite involving lesions of peripheral nerves e.g. polyneuropathies do not lead to adrenergic sensitivity (Baron, 2004). There is also evidence that patients with long-lasting painful neuropathy associated with inflammation lose the sympathetic maintained pain over time (Baron, 2004).

It seems that further research is needed to establish the mechanism of afferent adrenergic sensitivity in human neuropathies of different etiologies and in particular the time course during the disease process for the development of adrenergic sensitivity.

1.5.7 Pharmacological treatments of neuropathic pain

The most extensively studied drugs for the pharmacological treatment of neuropathic pain are antidepressants and anticonvulsants.
The tricyclic antidepressants exert their analgesic effect by modulating voltage-gated sodium channels but at lower doses, inhibit the reuptake of the monoamines noradrenaline and serotonin. These tricyclics have been effective in relieving pain in neuropathic pain states independently of the mood state of the patients so that the treatment was effective in both depressed and non-depressed patients (McQuay et al., 1996). Tricyclic compounds with mixed noradrenergic and serotonergic reuptake inhibition e.g. amitriptyline, imipramine and clormipramine have demonstrated greater therapeutic effects than the selective serotonin reuptake inhibitors such as paroxetine (Wolfe and Trivedi, 2004). The use of the tricyclics are limited because of their side effects which include urinary retention, cardiac arrhythmias, orthostatic hypotension and dizziness, effects mainly contraindicated in patients with cardiovascular problems and thus the elderly, who are more likely to suffer neuropathic pains (Wolfe and Trivedi, 2004).

Anticonvulsant medication such as carbamazepine and phenytoin have also been used for the management of neuropathic pain as a result of their membrane-stabilizing properties by blocking Na^+ channels and therefore reducing neuronal excitability (Sindrup and Jensen, 1999; Dickenson et al., 2002). Based on Na^+ channel blocker mechanisms, the use of topical medication with local anaesthetics for treatment of neuropathic pain such as lidocaine has benefits in providing a barrier which helps protect the area of hypersensitive skin of the damaged peripheral nerve (Galer et al., 1999).

Much interest has been focused on gabapentin (GBP), an anticonvulsant developed as a structural GABA analogue, yet despite its name, GBP has no direct GABAergic action nor any obvious effects on levels of the inhibitory transmitter. The mechanism of action of GBP is not clear, however several studies suggested an antagonistic

effect of GPB on alpha 2-delta subunit of voltage-dependent Ca^{2+} channels and this may explain the analgesic effect of this drug (Gee et al., 1996; Matthews and Dickenson, 2002). This subunit as well as certain calcium channels such as the N-type are upregulated after nerve injury and P-type after tissue damage (Dickenson et al., 2002). This may be an explanation of why GBP is effective in the presence of nerve injury or inflammation and yet has no effect in normal animals (Stanfa et al., 1997).

Another important class of drug for the treatment of neuropathic pain are opioids. Although studies done by Arner and Meyerson, (1988); Iadarola and Caudle, (1997) and Abdulla and Smith, (1998) indicated that morphine lacks full analgesic effects in neuropathic pain, it is now believed that opioids do have effectiveness but at higher doses (Dickenson and Suzuki, 2005), and there is further discussion of opioid treatments in neuropathic pain patients and animal models in chapter 3. This analgesic effect of morphine attracted attention of many research studies, and it appears that the efficacy of morphine in attenuating mechanisms of allodynia and or hyperalgesia, the classical symptoms of neuropathic pain, depends on the animal model and the route of administration of the drug, see Suzuki et al., (1999). This may explain some of the issues in patients where the syndrome and the symptom may well influence the effect of opioids.

Furthermore, the diverse mechanism and symptoms of neuropathic pain have drawn attention to the idea that combination therapy, based on multiple pharmacological targets and low drug doses, could improve both the pain and the side effect profile of treatments. In fact Matthews and Dickenson, (2002) demonstrated that combination of morphine and GBP had significantly improved effectiveness compared to morphine alone in a rat model of neuropathic pain.

There are other clinically licensed drugs that show potential for the treatment of neuropathic pain. These include NMDA antagonists, such as ketamine and memantine, although their use is limited by adverse side effects due to their global

block of NMDA receptor types (Dickenson et al., 2002). In addition studies also confirm the capacity for analgesic potentiation when NMDA receptor antagonist is added to morphine (Portenoy, 2000). Another important class for the treatment of neuropathic pain are the alpha 2-adrenergic agonists such as clonidine, further discussion see section 1.5.6.

1.6 Inflammation

Inflammatory pain is characterized by increased afferent inflow to the CNS from the injured area as a result of increased activity and responsiveness of sensitized nociceptors. Also nociceptive neurones in the spinal cord undergo changes which alter their responsiveness to input from the periphery. These changes lead the subject to experience spontaneous pain, hyperalgesia and allodynia, all conditions described in the neuropathic pain section but here, having very different causal mechanisms.

Inflammatory states such as arthritis and postoperative pain are associated with tissue damage and result from the release of complex mediators such as cytokines and other neuroactive agents, from both inflammatory and noninflammatory cells at the site of tissue damage (see Fig.4). These mediators include release of adenosine triphosphate (ATP) and protons from the damaged cells, which might directly depolarize primary afferent through activation of membrane cation permeability of P2X3 receptors or acid-sensing-ion-channels (ASICs) (Millan, 1999). Mast cells release amines such as 5-HT and histamine as well as arachidonic acid metablolites including prostaglandins. These prostaglandins are the prototype prostanoids and their synthesis is normally regulated by cyclo-oxygenase 1 (COX1) activity. However during inflammation large amount of prostanoids are synthesized due to the induction of COX2. In fact, due to the different properties of COX1 and COX2 enzymes, most non-steroidal anti-inflammatory drugs (NSAIDs) exercise their inhibitory effect on COX2 (Dray, 1997). Serotonin can cause direct excitation of sensory neurones by increasing Na^+ permeability via activation of 5-HT3 receptors or by decreasing K^+ permeability via G-protein coupled receptors such as 5-HT1 and 5-HT2 receptors, for

review see Dray, (1995). The degranulation of mast cell releases also histamine that acts on sensory neurones expressing H1 receptors and produce increase of membrane Ca^{2+} permeability likely to evoke release of neuropeptides as well as other pro-inflammatory mediators.

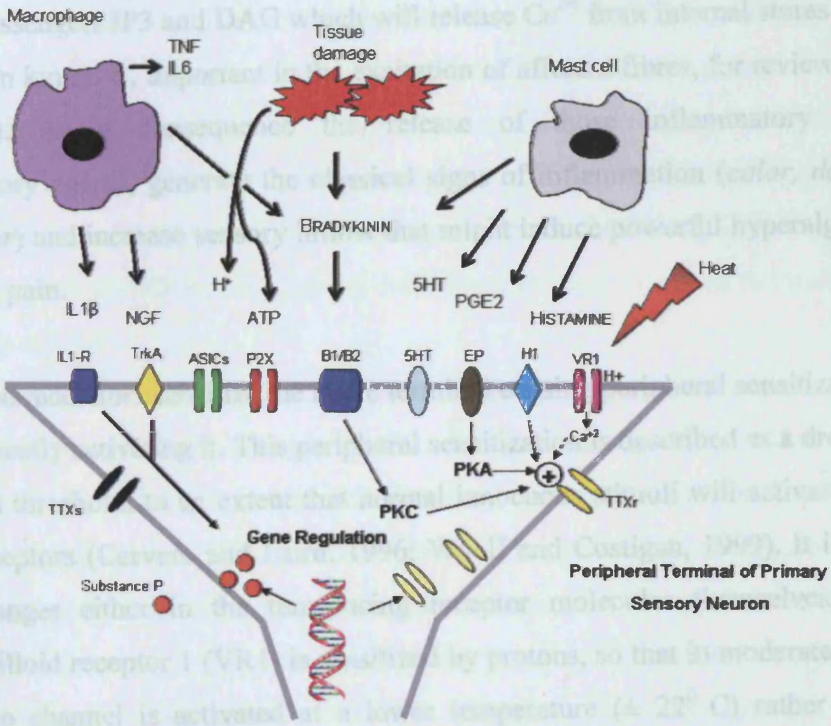

Figure4. Peripheral changes that occurs at peripheral terminasl of the primary sensory neurones in response to inflammation.

Furthermore, macrophages add to the inflammatory soup by releasing cytokines which includes interleukins (IL-1, IL-6), tumor necrosis factor (TNFα) and NGF. Bradykinin released from multiple cell types, exerts a number of proinflammatory effects mediated by two distinct receptors B1 and B2. The B2 receptors, the most studied receptors, are coupled with G-proteins to phospholipase C, thus generating the second messengers IP3 and DAG which will release Ca^{2+} from internal stores and activate protein kinase C, important in the excitation of afferent fibres, for review see Dray, (1995). As a consequence the release of those inflammatory and noninflammatory agents, generate the classical signs of inflammation (*calor, dolor, tumor or rubor*) and increase sensory inflow that might induce powerful hyperalgesia and perceived pain.

However, most mediators sensitize the nerve terminal causing peripheral sensitization rather than directly activating it. This peripheral sensitization is described as a drop in the peripheral threshold, to an extent that normal innocuous stimuli will activate the sensitized receptors (Cervero and Laird, 1996; Woolf and Costigan, 1999). It is the result of changes either in the transducing receptor molecules themselves, for example, vanilloid receptor 1 (VR1) is sensitized by protons, so that in moderate acid conditions the channel is activated at a lower temperature (± 22^0 C) rather then intense heat stimulus (> 45^0 C). Also it has been seen that Na^+ channels are other contributors to peripheral sensitization. Here the phosphorylation of the TTXr Na^+ channel by protein kinase A (PKA) and PKC increases Na^+ channel currents in the terminals and subsequently by lowering the threshold of the neurone, a depolarizing stimulus will produce a greater excitation (Fitzgerald et al., 1999; Gold, 1999) see Fig.4.

These different chemical mediators not only initiate various signal transduction cascades that result in immediate changes as above described, but the response to inflammation is further amplified by later changes in gene transcription (Woolf and Costigan, 1999). This gene transcription will reinforce the inflammatory cascade by

means of changes in expression and transport of proteins in the peripheral terminal (Fig.4) and in the DRGs of the neurones as well as dorsal horn neurones.
Example of the transcriptional changes are as follows; up-regulation of neuropeptides such as substance P that is transported back out to the peripheral terminal. This peptide as well as CGRP, induces vasodilatation which allows movements of protein and antibodies as a result of plasma extravasation and mast cell degranulation that will further promote the inflammatory process (Dray, 1995; Costigan and Woolf, 2000). Similarly to substance P, NGF, acting via a specific tyrosine kinase receptor (TrkA), presents a key step in the initiation of gene transcription. Its upregulation, thus further increases the synthesis of several neuropeptides including CGRP (Dray, 1995), which causes vasodilatation together with its synergism effect on substance P as seen in section 1.5.5.1.

Furthermore, related to these changes is the upregulated expression of several channels such as VR1, TTXr, that may increase neuronal response following inflammation (Costigan and Woolf, 2000). Overall these inflammatory cascades cause sensitization of peripheral nociceptors and subsequently change their response properties such as generation of spontaneous activity which will provide a continuous spontaneous pain and a decrease in threshold. Moreover, these changes not only evoke excitation of the normal nociceptors but also recruit silent nociceptors (Dray, 1995; Cervero and Laird, 1996).

In addition to the peripheral changes of inflammation, changes in the expression of neuropeptides and receptors transported to the central terminals of the neurones can amplify the central actions of the transmitters released from sensory neurones. Therefore, levels of neuropeptides and aminoacids such as glutamate, substance P and CGRP are upregulated and also expression of large molecules with modulatory roles such as brain derived neurotrophic factor (BDNF) are increased in the dorsal horn of the spinal cord (Woolf and Costigan, 1999). As results of these changes, multiple central receptors will be activated and increases in intracellular events such as Ca^{2+}, both via Ca^{2+} influx and release from intracellular stores and a consequent

activation of Ca^{2+} dependent enzymes including PKC, PKA, TrkA receptors will initiate multiple signal transduction cascades (Costigan and Woolf, 2000). These may target several receptors of which NMDA and AMPA are the prototypes, and cause changes in neuronal properties in response to noxious and innocuous stimuli, and therefore initiate central sensitization as well as altering the phenotype of sensory neurones (Neumann et al., 1996).

As a consequence, the neuronal excitability of the dorsal horn will increase and become more excitable in response to stimuli of lower intensity and also some lower-threshold A-fibres acquire the chemical phenotype typical of C-fibres. Furthermore, changes in neuronal response will be altered also in the uninjured area surrounding the injured site, a phenomenon known as secondary hyperalgesia which is thought to be mediated by plasticity of central synaptic processes and therefore related to central sensitization (Schmelz et al., 2000).

A variety of other substances are released following inflammation, these involve the reactive oxygen species (Dray, 1995) including hydrogen peroxide which enhances the effect of other mediators such as bradykinin and prostaglandin while NO prior to its contribution in mechanisms involving increase of neuronal excitability as discussed in section 1.5.5.1, may alter the responsiveness of sensory neurones to inflammatory mediates such as bradykinin (Dray, 1995).

The other family of spinal amino acids altered in this inflammatory process is the group of the inhibitory neurotransmitters such as GABA, since the levels of this neurotransmitter are increased in the dorsal horn (Castro-Lopes et al., 1994). The endogenous opioid systems also undergo changes that will be discussed in chapter 3.

As a result, all of these changes may contribute to alteration or modulation of nociceptive transmission in the periphery and central nervous system and further enhance and prolong the inflammatory pain state.

1.6.1 Choice of animal model for inflammation

The model of inflammation used in the thesis was chosen as a model where the inflammation can be induced in a short time, in hours rather than days or weeks. This short time scale of the development of inflammation is important to allow study of the neuronal response from a control period pre-inflammation and through to the peak response of hyperalgesia. These criteria exclude models of chronic polyarthritis, where the intradermal injection of complete Freund's adjuvant (CFA; emulsion containing inactive mycobacterium butyricum) at the tail, causes inflammation which reaches a peak level only after 3 weeks (Millan et al., 1986). Another inconvenience of this model is the generalized effect of this disease which involves changes in many organs and systems, not necessarily related to pain. In order to reduce the duration of the stable maximum inflammation effect and to restrict the area of inflammation, models of unilateral inflammation produced by intradermal injection of CFA into the plantar surface of the animal's paw was considered. However the hyperalgesia produced in this model reaches the maximum peak 1-6 days after the injection (Iadarola et al., 1988), and therefore was not suitable for my study.

An interesting model of peripheral inflammation is the formalin test; in this model a dilute solution of formalin was injected into the hind paw and induces two phases of nociceptive behavioural which lasts for 1 h, for review see Tjolsen et al., (1992). The first phase, starting immediately after the injection of formalin and lasts for up to 5 min, is thought to be mediated by direct chemical stimulation on C-fibres evoked activity (Dickenson and Sullivan, 1987b; Tjolsen et al., 1992). Subsequently, there is a second phase which starts approximately 10-15 min after the injection and lasts for 20-40 min, and it is believed that this phase is mediated by inflammatory reaction since NSAIDs reduce nociceptive behavioural responses during this phase, while the first phase seems unaffected. Both phases cause functional changes in the dorsal horn of the spinal cord, moreover the pain behaviour displayed in these animals consists of licking and biting the injected paw and lasts one hour. Similarly electrophysiological

studies of this acute inflammatory model reveal this characteristic bi-phasic pattern of firing (Dickenson and Sullivan, 1987b) which correlates to the level of pain behaviour as well as the timing of the reactions in the behavioural studies. However, one limitation of this model is the short duration of the inflammation, and also the ongoing firing of the dorsal horn neurones evoked by this model complicates the investigation of the central mechanism of hyperalgesia which accompanies inflammatory pain state.

For these reasons, in these experiments was used a model of inflammation induced by subcutaneous injection of carrageenan into the hind paw of the rat. This model described in section 2.3 of methods, was first proposed by Winter et al., (1962), since then this model has been widely used as a model of acute cutaneous hyperalgesia, where animals develop hyperalgesia to mechanical and thermal stimuli without any indicative spontaneous pain behaviour. The signs of inflammation may already be seen at 1 h after the intraplantar injection of carrageenan and the inflammation is stabilized between 3 and 4 h, and largely resolved within 24-96 h (Kayser and Guilbaud, 1987).

1.7 Aims of this thesis

The aim of this thesis was to investigate the role of the nociceptin receptor system in different pain states using responses of spinal dorsal horn neurones. Moreover, since the pharmacology of this receptor is still under study, with few molecules selectively acting on this receptor, it was interesting to study the potential of agonists and antagonists at this receptor. One major aim was to determine if the antagonist could reveal any endogenous activity of nociceptin at the ORL_1 receptor in the different pain models.

Since CCK is believed to be involved in processes that lead to a reduction in morphine analgesia, it is important to study any interaction between CCK and the ORL_1 receptor to determine if this receptor is modulated in the same way as the mu receptor.

In addition, taking into account the importance of lamina I neurones in the control of pain I wished to understand the influence if any of the ablation of lamina I NK1 expressing neurones on the function of the nociceptin system on spinal processing and to compare with an opioid agonist acting on the delta receptor. The delta opioid receptors are present predominantly at pre-synaptic sites on peripheral terminals of afferent neurones and therefore it was important to study their effects in animals with ablated lamina I neurones expressing NK1 receptors which would remove post-synaptic opioid receptors found on these neurones. Moreover, delta receptors are appropriate to compare with ORL-1 receptors since both receptors are not the main opioid receptors in the spinal cord.

Finally, it was also my aim to study the spinal effect of oxytocin in normal and neuropathic animals.

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CHAPTER 2

METHODS

All experiments were carried out on male Sprague-Dawley rats obtained from University College London Biological Services. Animals were housed 4 or 5 per cage with a 12 h: 12 h light: dark cycle, water and food *ad libitum*. All procedures were conducted in accordance with the U.K. Home Office legislation for animal experimentation and the International Association for the Study of Pain (Zimmermann, 1983). Animals used in these experiments were divided into, normal, operated and carrageenan injected rats. The operated rats were subject to either: i) spinal nerve ligation (SNL), ii) sham operation for SNL surgery or iii) intrathecal SP-SAP injection, to produce an ablation of Lamina I spinal neurones expressing substance P receptor.

2.1 Spinal nerve ligation (SNL)

Male Sprague-Dawley rats initially weighing 130–150 g, were used for SNL surgery. Selective tight ligation of the spinal nerves L5 and L6, was performed as first described by Kim and Chung, (1992). Animals were divided into a control group (sham operation) and an experimental neuropathic group (SNL).

The animals were anaesthetised with a mixture of halothane (3.5% for induction, 1.5% for maintenance) and 50% N_2O: 50% O_2. The rat was placed in a prone position. A midline incision of 2 cm length was made from L4-S2 and the left paraspinal muscles were separated from the spinal processes. The L6 transverse process and the sacrum were made visible by scraping off the attached ligaments. Part of the L6 transverse process was then removed to expose the L4 and L5 spinal nerve and L6 was identified as lying just under the sacrum. Using a 6-0 silk thread, the left spinal nerves L5 and L6 were tightly ligated distal to the dorsal root ganglion and proximal to their conjunction to form the sciatic nerve. Haemostasis was confirmed, the wound was closed using 3-0 surgical sutures and wound clips.

Sham operations were performed and the surgical procedure was identical to that of the experimental group, with the exception of the spinal nerve ligation. Only animals

without impaired motility and showing mechanical and with satisfactory behavioural responses such as mechanical and cold allodynia were used in the experiments.

2.1.1 Behavioural studies

After the surgery, animals were housed under the same condition as before the preoperative period. The general behaviour of the animals was closely observed, and their weights monitored.

Successful reproduction of the neuropathic model was confirmed by behavioural testing performed postoperatively on days 2, 4, 8, 11 and 14. Animals were placed individually in transparent cages with a wire mesh bottom that allowed full access to the paws.

The mechanical sensitivity of the ipsilateral and contralateral hindpaw was assessed by measuring the frequency of withdrawal of the foot to a normally innocuous mechanical stimulus. The stimulus was applied, from below the mesh floor, to the plantar surface of the foot with three different von Frey filaments (1, 5 and 9 g) until reaching the bending forces. Each trial consisted of the application of a single von Frey hair 10 times, each for a period of 2-3 sec. Using ascending and consecutive von Frey hairs each trial was separated by a period of at least 3 min. The number of withdrawals to each force was counted.

Cooling sensitivity was determined using a drop of acetone applied onto the dorsal region of the rat's foot using a 1 ml syringe connected to polyethylene tube (polyethylene; id. 1.57 mm; od. 2 mm). In order to avoid the mechanical response caused by the tube, particular attention was taken to not touch the foot of the rat while applying the acetone drop. Each test consisted of 5 applications and each trial was separated by a period of 5 min.

Spinal nerve ligated animals demonstrated enhanced responses to both mechanical and cooling responses on the ipsilateral hind paw (Fig.1). These responses consisted of licking and shaking of the hindpaw. No withdrawal response was observed on the contralateral hind paw of SNL rats and on both sides in sham operated rats. These behaviours confirm the success of the surgery and the enhanced withdrawal response to innocuous stimuli was interpreted to be a sign of allodynia.

The response to both mechanical and cooling stimuli was quantified and expressed as different score:

Difference Score = number of foot withdraws on contralateral paw – number of foot withdrawals on ipsilateral paw.

For example, if the difference score is a value close to zero (1, 0 or -1) then there is no difference between the contralateral and the ipsilateral paw and this was the case for sham operated animals; while in SNL animals the difference scores are more negative values (from -2 to -6) indicating a greater foot withdrawal response on the ipsilateral hindpaw and was interpreted to be a manifestation of mechanical or cold allodynia (Fig.1).

The behavioural tests show stable allodynia from 2 days after surgery and this gradually increased in magnitude and persisted over the 2 week testing period, see (Fig.1).

Two week after the postoperative period, both SNL and sham operated animals were ready for the electrophysiology studies.

2.2 Ablation of lamina I spinal neurones expressing NK1 receptor

The procedure of ablating lamina I spinal neurones expressing the receptor for substance P was first described by Mantyh et al., (1997) and involves intrathecal administration of a cytotoxin conjugated to the ligand, SP (SP-SAP). In these experiments, rats weighing 130–150 g were anesthetized with a combination of

intraperitoneal Diazepam (2.5 mg/kg, Phoenix Pharmaceuticals, UK) and intramuscular Hypnorm (0.3 ml/Kg, Janssen Animal Health, Belgium), and placed in a stereotaxic frame.

A small incision (1 mm long) was made in the atlanto-occipital membrane, followed by insertion of a cannula (8.5 cm, polyethylene, id x od, 0.28 X 0.61 mm) into the subarachnoid space. The cannula was used to deliver the conjugate at the lumbar (L4-L5) level. 10 µl of either saline or 10^{-6}M SAP or 10^{-6}M SP-SAP (Advanced Targeting Systems, San Diego, USA) was injected, followed by a 5 µl saline flush using a 50 µl Hamilton syringe. Five min after the intrathecal injection, the cannula was carefully removed and the wound was closed using 3-0 surgical sutures and wound clips.

Animals were housed in groups of 3-4 per cage and their behaviour was closely observed. Animals were used for the subsequent electrophysiology studies, 28 days after injection.

2.3 Carrageenan

First described by Winter et al., (1962), carrageenan, a substance used to induce inflammation is stable at $\geq$ 3 h after subcutaneous injection into the plantar surface of the hindpaw.

In this study, after a neurone was found 100 µl of carrageenan 2% was injected into the plantar surface near the receptive field of the neurone. Controls were taken throughout the 3 h period following carrageenan injection after which pharmacological studies were commenced.

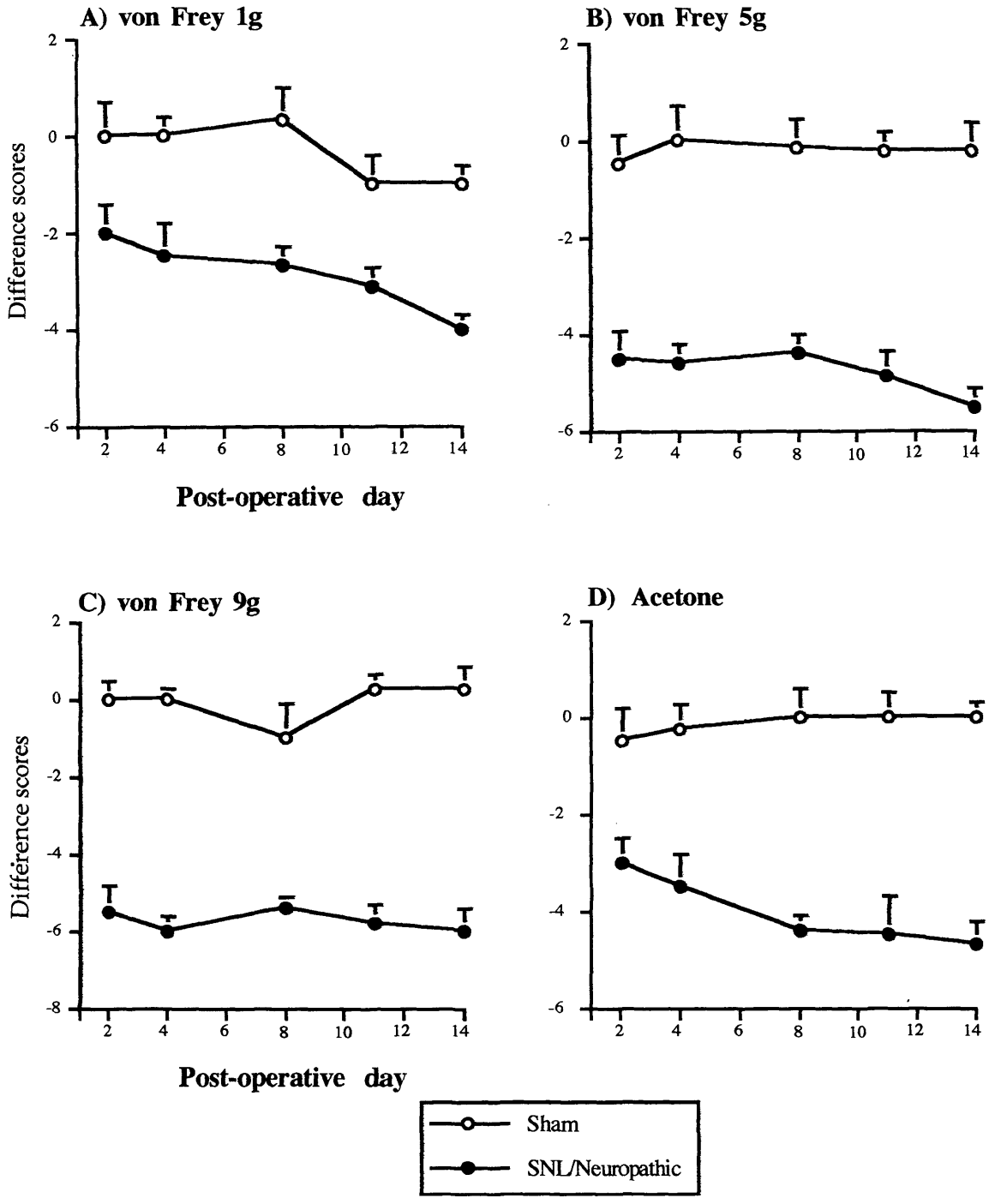

Figure 1. The difference score of foot withdrawal frequencies to the application of A) von Frey 1g, B) von Frey 5g, C) von Frey 9 g and D) acetone in sham operated (n= 24) and SNL/neuropathic (n= 24) rats. In all SNL rats there is a higher frequency of foot withdrawal on the ipsilateral hindpaw and this indicates the present of allodynia in these animals. The allodynia was observed from day 2 and gradually increased throughout the testing period (14 post-operative days).

2.4 Electrophysiology recordings

This electrophysiological study follows the procedure previously described by Dickenson and Sullivan, (1986). For these studies, several animal groups were used including normal animals (weighing 200–250 g), those that had been operated for spinal nerve ligation, sham operation, or SP-SAP treated rats (weighing 350–400 g).

Rats were initially anaesthetised with 3.0–3.5% halothane in a gaseous mixture of 66% N_2O: 33% O_2 and maintained throughout the experiment via a cannula (polyethylene tube; id. 1.57 mm; od. 2 mm) inserted into the trachea. The rats were placed in a stereotaxic frame and secured by ear bars to maintain stability during electrophysiological recordings. The vertebrae rostral and caudal to vertebra L1–L3 were clamped and a laminectomy was performed to expose L4–L5 spinal cord segments, which receive afferent input from the toe regions of the rat. Subsequently, the level of halothane was reduced to 1.0–1.5%. The body temperature of the rat was maintained at 37 ^{0}C with the heating blanket connected to a rectal probe via an automatic feedback control unit. A parylene coated tungsten electrode was descended into the cord using a manipulator and extracellular recordings were made from single wide dynamic range neurones. The depth of the recording site was noted from the microdrive reading connected to the manipulator.

Extracellular recordings were made from single dorsal horn neurones that either receive input from the toe region ipsilateral to the spinal nerve ligation or sham procedure, or from either side of spinal cord in normal and saline/SAP/SAP-SP rats.

The selected neurone must respond to both noxious and non-noxious stimuli. Two fine needles, attached to a stimulus isolator module, were applied to the receptive field, to allow transcutaneous electrical stimulation. The C-fibre threshold was determined by giving a single, electrical pulse (amplitude 0.1–3.3 mA) incrementally until a C-fibre latency response was evoked. Tests consisted of a train of 16 electrical stimuli (0.5 Hz, 2 ms pulse duration) given at 3-times the threshold required to evoke

a C-fibre response. The action potentials evoked by each stimulus were superimposed and constructed into a post-stimulus time histogram (PSTH) by the Spike2 software (Fig.2). These were then separated on the basis of latency, into total Aβ-fibre (0–20 ms)-, Aδ-fibre (20–90 ms)- and C-fibre (90–300 ms)-evoked action potentials. The remaining neuronal responses recorded (300-800 ms post-stimulus) were classed as 'Post-discharge' while input and wind-up responses were calculated as follows:

Input = (Action potentials recorded 90-800ms evoked by first stimulation) x (total number of stimuli (16)).

Wind-up = (Total action potentials recorded 90-800ms after the train of 16 stimulation) – Input.

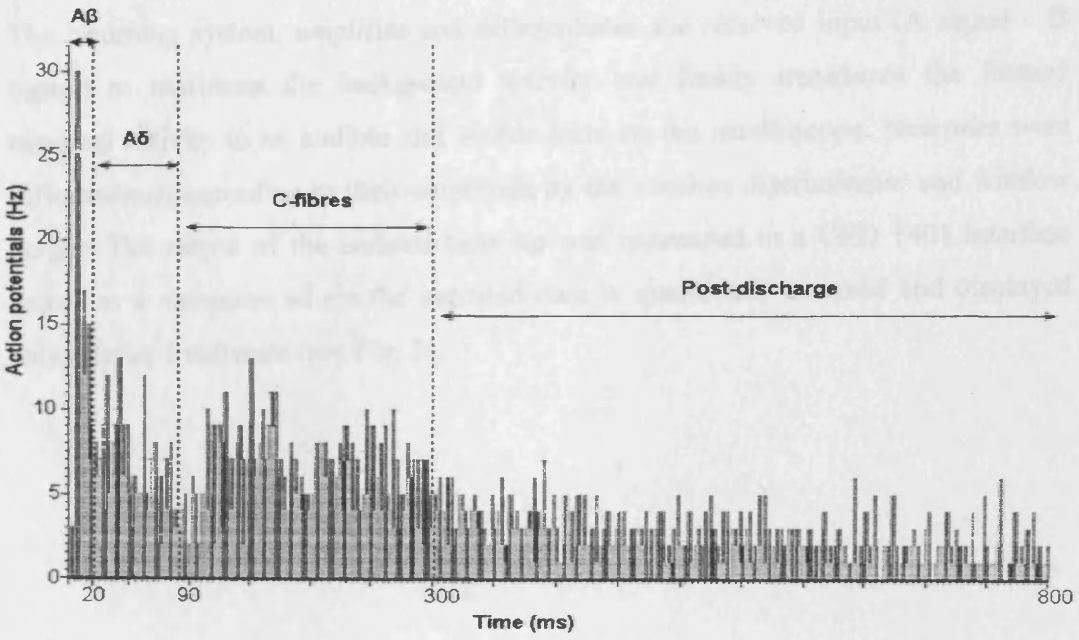

Figure 3. A typical post-stimulus time histogram (PSTH) obtained from a single dorsal horn neurone showing the responses produced by the 16 electrical stimuli. Latency bands for Aβ-, Aδ- and C-fibre-evoked responses and post-discharge are indicated.

2.4.1 Data acquisition in the electrophysiology recordings

The headstage receives input from the recording tungsten electrode (A-signal), which contains the response from the neurone, background activity of the spinal cord and electrical interference of the surrounding environment. A second input (B signal) was received by the headstage, which monitored activity from the cardiovascular and respiratory system of the animal as well as an eventual electrical interference of the surrounding environment. Finally the headstage receives also input from a lead connected to the stereotaxic frame (earth), grounded prior to recordings.
The headstage, is attached to a manipulator which regulates the course and fine movements of the electrode and the output is sent to a Neurolog system.

The Neurolog system, amplifies and differentiates the received input (A signal – B signal) to minimize the background activity and finally transduces the filtered neuronal activity to an audible and visible form on the oscilloscope. Neurones were differentiated according to their amplitude by the window discriminator and window height. The output of the isolated neurone was connected to a CED 1401 interface linked to a computer where the captured data is quantified, analysed and displayed using Spike 2 software (see Fig. 3).

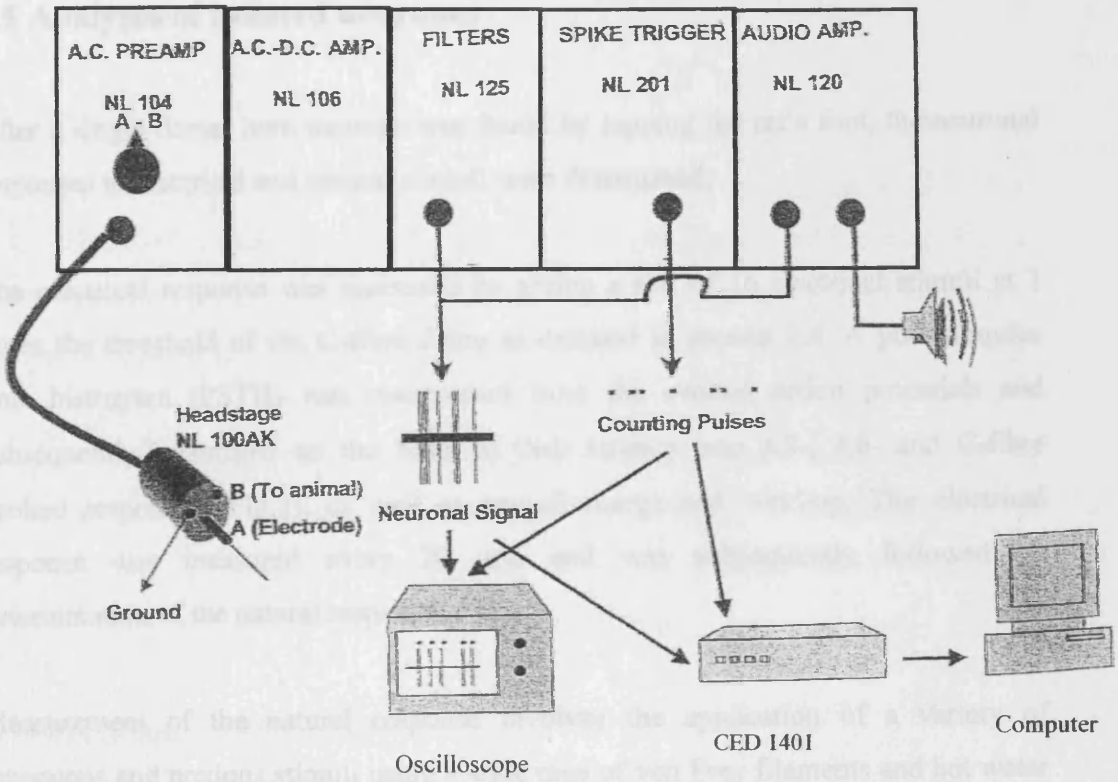

Figure 3. Schematic representation of neuronal recording system. Adapted from (Stanfa and Dickenson, 2004).

2.5 Analyses of isolated neurones

After a single dorsal horn neurone was found by tapping the rat's foot, the neuronal responses to electrical and natural stimuli were determined.

The electrical response was measured by giving a test of 16 electrical stimuli at 3 times the threshold of the C-fibre firing as detailed in section 2.4. A post-stimulus time histogram (PSTH) was constructed from the evoked action potentials and subsequently quantified on the basis of their latency into Aβ-, Aδ- and C-fibre evoked responses (Fig.2), as well as post-discharge and wind-up. The electrical response was measured every 20 min and was subsequently followed by determination of the natural response.

Measurement of the natural response involves the application of a variety of innocuous and noxious stimuli using a wide rage of von Frey filaments and hot water jets. Von Frey filaments were applied in an ascending order (2, 5, 9, 15, 30, and 75 g) onto the central receptive field of the neurone, each one applied for 10 s. Jets of water ranging in temperatures 32, 35, 40, 42, 45, 48 and 50° C were delivered from a 50 ml syringe. The spontaneous activities of the neurones were recorded prior to the application of natural stimuli over a period of 5 min or until response stabilization. The ongoing activity was then subtracted from the evoked response of the individual test. An interval of 2-3 min was allowed between each electrical, thermal and mechanical test. In some experiments, only responses to electrical stimulation were determined and tests were conducted every 10 min.

2.6 Drug application

Control values were taken for each test until a minimum of three consecutive stable values (≤ 10% variation) were obtained, this was then followed by the application of a drug. Most of the drugs studied in this thesis were applied intrathecally, directly onto the exposed spinal cord using a 50 µl Hamilton syringe, while others were injected subcutaneously into the scruff of the neck.

Drugs were administered in cumulative doses, where each dose was followed for 20-60 min in order to obtain a maximum effect, which normally occurred between 40-60 min post drug application. The drug dose range was selected based on previous electrophysiological or relevant pharmacological studies.

2.7 Statistical analysis of results

The three electrically evoked neuronal responses taken immediately prior to the drug administration were averaged and used as controls (pre-drug control value) to calculate the subsequent drug effects. The effects of drugs on the electrically evoked responses were then calculated as percentage of pre-drug control values (% control). I then determined the maximal change (largest increase or decrease from the control value) for each dose of drug in each experiment. The results for each dose were expressed either as mean % control or % inhibition (where the % inhibition =100 - % change based on the control value, i.e. 80% control=20% inhibition) ± standard error of mean (S.E.M.). Also the effects of the drugs on the natural evoked responses were expressed as mean number of action potentials evoked over 10 sec ± S.E.M.

Statistical analysis of drug effects at each dose compared to the pre-drug control values was assessed using repeated measures analysis of variance (ANOVA) as well as Tukey test on raw data. Comparison of drug effects between different experimental groups (normal, sham and neuropathic/SNL) was made using two way ANOVA and Tukey test on raw data. The level of significance was taken as $P \leq 0.05$. The only

additional analysis was a Mann-Whitney test used for the particular experiments in Chapter 3, 6 and are described in that section. Statistical analysis of the data was performed using the programme InStat (GraphPad) installed on iMac computer.

2.8 Drugs employed

Nociceptin was obtained from Tocris. The peptide was dissolved in 0.9% saline solution.

J113397 was obtained from Banyou Pharmaceuticals, Japan. J113397 was dissolved in 0.9% saline and a drop of hydrochloric acid.

DPDPE was obtained from Tocris. The peptide was dissolved in 0.9% saline solution.

Oxycodone was obtained from Sigma and was dissolved in 0.9% saline solution.

Compound 20700857 was obtained from Solvay Pharmaceuticals. Due to solubility difficulties in saline, this was dissolved in a mixture of 1% methylcellulose and 5% mannitol.

CCK (Octapeptide Sulfate) was obtained from Tocris and was dissolved in 0.9% saline solution.

Oxytocin (Alpha-hypothamine) was obtained from Sigma and was dissolved in 0.9% saline solution.

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CHAPTER 3

THE SPINAL EFFECT OF NOCICEPTIN AND OXYCODONE

3.1 INTRODUCTION

3.1.1 Opioids

The endogenous inhibitory opioid system has been manipulated to provide pain relief since the discovery of opium, of which morphine is the main active component. Opioid receptors, known for over 30 years were originally divided into mu (μ), delta (δ) and kappa (κ) opioid receptors, for review see Pasternak, (1993). These receptors have been cloned and demonstrate a high degree of sequence homology (see Table 1 for opioid receptors, their ligands, and physiological effects).

The opioid receptors are present both in the central and peripheral terminals of the thinly myelinated and unmyelinated sensory fibres, as well as within the peripheral and higher centres of the nervous system, for review see (Sawynok, 2003). In the spinal cord opioid receptors are mostly located in the superficial dorsal horn (lamina I and II). The contribution of μ, δ and κ receptors to the total opiate binding throughout the spinal cord was estimated at 70%, 24% and 6%, respectively (Besse et al., 1990). However, this estimation was made prior to the discovery of the fourth opioid receptor.
Each receptor class exists in multiple subtypes; μ_1 and μ_2, δ_1 and δ_2 and κ_1, κ_2 and κ_3 (Pasternak, 1993) according to some but as yet, there is no evidence from molecular studies of any subtypes.

Up to at 70% of these opioid receptors are found presynaptically on the central terminals of primary afferent fibres, and they are expressed by small diameter nociceptive primary afferents (Aδ- and C-fibre). However, the remaining 30% are postsynaptic receptors that appear to hyperpolarize the dendrites of projection neurones and interneurones, also they could disinhibit inhibitory interneurones with the result of further inhibition of C-fibre transmission, for review see (Dickenson, 2001).

Opioids are used to relieve moderate to severe pain whatever the cause (accidents, pre- intra-post-operative pain, cancer, etc).

Table 1. Opioid receptors, their preferred ligands and physiological effects.

	Mu (μ)	**Delta (δ)**	**Kappa (κ)**
Endogenous agonist	endorphins	enkephalins	dynorphins
Exogenous agonists	morphine DAMGO	(morphine) DPDPE	(morphine) nadoline oxycodone
Antagonist	naloxone β-FNA	naloxone naltrindole	naloxone nor-BNI
Physiological effects	analgesia, ↓GI motility, respiratory depression, sedation, nausea and euphoria	analgesia sedation	analgesia dysphoria

DAMGO = [D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin; DPDPE = [D-Pen^2,D-Pen^5]-enkephalin; β-FNA = Beta-Funaltrexamine; nor-BNI = Norbinaltorphimine; ↓GI motility = reduced gastrointestinal motility. Morphine produces most effects through mu receptors (Kieffer, 2000) but has some affinity for delta and kappa and so is denoted as (morphine) for these receptors.

The mu-opioid receptors are thought to be responsible for most of the analgesic effects of opioids and for some major unwanted effects (e.g. respiratory depression, euphoria, sedation and dependence) (Kieffer, 2000).

One strategy to produce opioid-mediated analgesia whilst avoiding the side effects attributed by opiate receptors in the brain and periphery is to target spinal action of opioids by using the intrathecal or epidural routes of administration.
Spinal application of morphine has been demonstrated to reduce the release of a number of transmitters in the spinal cord including substance P, CGRP, glutamate and aspartate, which are normally released after noxious stimulation (Yaksh et al., 1980). This corresponds to one of the major mechanisms of action of opioids, through opioid receptor interaction with G-protein coupling (Gi or Go) to decrease adenosine 3,5-monophosphate (cAMP), increase K^+ efflux and decrease Ca^{2+} entry. These mechanisims attenuate the propagation of action potentials and release of neurotransmitters in the sensory nerve fibres (Sawynok, 2003).

Opioids also act at supraspinal levels, as injection of morphine into the PAG region causes marked analgesia, which can be prevented by surgical interruption of descending pathways to nucleus raphe magnus (NRM) or by blocking 5-HT synthesis (Fields et al., 2006). This last procedure interrupts the 5-HT pathways running from the NRM to the dorsal horn (Fields et al., 2006), however the mechanism of action of opioids at the supraspinal level are still poorly understood. It has been suggested that the descending controls filter sensory messages at the spinal level allowing a pain message to be extracted from the incoming message (Dickenson, 1994) and therefore supraspinal morphine is thought to reduce these controls and alter the perception of pain.

Opioid receptors are also present in the peripheral nervous system where they mediate analgesic effects similar to those observed at the spinal level (Stein et al., 2001). Despite the widespread use of opioids in the management of "nociceptive" pain, their effects are particularly altered under inflammatory conditions or pain arising from neuronal damage.

During inflammatory processes, opioid receptors transported from DRG towards the peripheral sensory nerve endings are enhanced (Hassan et al., 1993; Stein et al.,

2001). Thus, activation of these receptors can cause attenuation of the excitability of nociceptive input terminals, inhibit the release of neurotransmitters and ultimately result in increased analgesia. In addition to the increased receptor density, inflammation induces disruption of the perineurial barrier around the primary afferent that allows exogenous and also endogenous opioids released from immune cells to activate opioid receptors that are preexistent on sensory nerves, but inactive or inaccessible by the perineurial barrier under normal conditions (Antonijevic et al., 1995). Moreover, it has been speculated that a decrease in the level of spinal CCK during inflammation could be the basis for the increased morphine potency in this pain state (Stanfa and Dickenson, 1993; Stanfa et al., 1994). All these changes may interact to enhance the inhibitory effect of opioids in presence of inflammation. In accordance with those hypotheses, several studies demonstrated that morphine and other opioid agonists are more efficacious as analgesic agents in animals with inflammatory condition than in normal animals (Stanfa and Dickenson, 1993; Ossipov et al., 1995).

Another pain state where the effect of opioid is a matter of considerable controversy is presented by neuropathic pain. Neuropathic pain as described previously in chapter 1, was found in some studies to not be adequately treated by opioids (Arner and Meyerson, 1988; Iadarola and Caudle, 1997; Abdulla and Smith, 1998). The reduction of opioid sensitivity may be caused by a reduction in the number of spinal opioid receptors as studies have shown that mu-opioid receptors expression is mainly downregulated after nerve injury (Porreca et al., 1998; Zhang et al., 1998). Further, increased CCK (for discussion see chapter 6) and the excessive spinal hyperexcitability after repeated C-fibre stimulation which is hard to inhibit (Dickenson, 2001) may participate in the reduced opioid sensitivity in this pain state.

In addition to those factors, numerous studies suggested that antinociceptive abilities of morphine in behavioural studies on neuropathic animals depends on variables such as methods of behavioural assessment, nature of the stimuli utilized as well as the route of administration and the neuropathic model used, see (Suzuki et al., 1999) for

further discussion. Thus, it appears that all these variables may explain the different sensitivity of opioids in neuropathic pain state and so it is now believed that opioids do have effectiveness but at higher doses (Dickenson and Suzuki, 2005). This may also be translated into higher side effects of opioids.

Therefore all attempts to separate the analgesic properties of opiates from their other CNS effects, the clinically problematic respiratory depression, nausea and sedation, or the perceived problems of dependence and euphoria, have been unsuccessful. All these effects, as well as the peripherally mediated constipatory effects of morphine are mediated via the mu- opioid receptor (Kieffer, 2000). In an effort to avoid these unwanted central effects, agents selective for the delta- and kappa-opioid receptors have been investigated as potential analgesics, but with limited success. It was the search for other more 'pain' specific opioid receptors (or subtypes) that led to the discovery of the orphan opioid receptor-like (ORL_1/NOP) receptor (Mollereau et al., 1994; Wang et al., 1994). The following section will be the basis for the study of ORL_1 agonists and also Oxycodone.

3.1.2 Nociceptin (N/OFQ)

The use of molecular biology approaches led to the discovery of a novel receptor identified as opioid-like receptor 1 (ORL_1) receptor (Mollereau et al., 1994; Wang et al., 1994) whose ligand is the heptadecapeptide (FGGFTGARKSARKLANQ) known as Nociceptin or Orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995). The term of orphanin FQ refers to its affinity for the "orphan" opioid receptor, while the F and Q refers to the first and last amino acids, phenylalanine and glutamine.

The ORL_1 like other opioid receptors is a G-protein-coupled receptor with seven predicted transmembrane domains (see Fig.1). This receptor shows overall 60% homology with the other opioid receptors and >90% sequence identities between

species variants; human, rats and mouse (Calo et al., 2000; Mogil and Pasternak, 2001).

As shown by several groups, activation of ORL_1 by nociceptin inhibits forskolin-stimulated cAMP accumulation, activates K^+ channels and inhibits voltage-dependent Ca^{2+}current in a similar way to the other three opioid receptors (Abdulla and Smith, 1998; Hawes et al., 2000).

In spite of the morphological similarity between the 'classic' opioid receptors and ORL_1, there is no pharmacological homology. In fact, none of the existing endogenous opioid-peptide agonists have significant affinity for the ORL_1-site, except dynorphin A (Henderson and McKnight, 1997). Naloxone, a non selective opioid receptor antagonist showed very little affinity, if any, for ORL_1 (Mollereau et al., 1994).

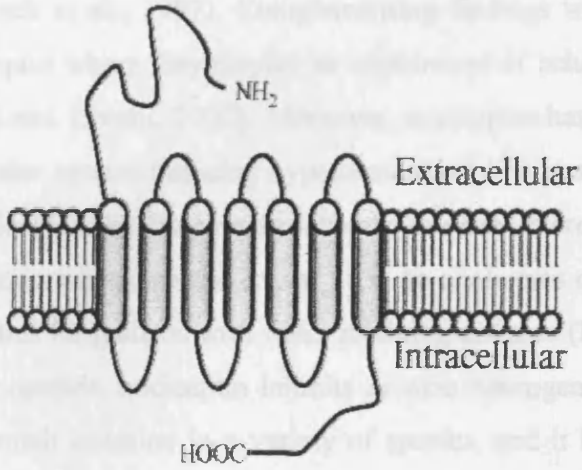

Figure 1. Structure of ORL1 receptor, a G-protein coupled receptor with seven transmembrane domains. The upper part (exrtacellular) contains N-terminal while the C-terminal side is in the intrsacellular. Picture adopted by (Mogil and Pasternak, 2001).

3.1.2.1 Distributions and biological actions of nociceptin

The distribution of nociceptin and its receptor in the central and peripheral nervous system has been investigated with in situ hybridization and immunohistochemical techniques. Both the receptor and the peptide are widely expressed in brain areas, such as amygdala, hypothalamus, PAG, dorsal raphe nucleus and spinal cord dorsal horn suggesting its involvement in nociceptive processing and also in other general functions (Calo et al., 2000; Mogil and Pasternak, 2001).

Of the many proposed functions of nociceptin, an involvement in learning and memory has received particular attention. Knockout mice lacking the ORL_1 receptor have been reported as showing improved learning and memory, suggesting that nociceptin has inhibitory functions (Mamiya et al., 1998). Nociceptin and its receptor are also involved in emotional and stress regulation, as number of standard behavioral assays reveal the ability of supraspinal nociceptin to block fear and anxiety in both rats and mice (Jenck et al., 1997). Complementing findings were made with mice lacking ORL_1 receptor where they display an impairment of behavioural responses to stress (Reinscheid and Civelli, 2002). Moreover, nociceptin has an inhibitory effect on the cardiovascular system inducing hypotension and bradycardia in rats (Giuliani et al., 1997). Nociceptin also induces an increase in water excretion and decrease in urinary Na^+ excretion when injected i.v. or i.c.v. so analogues of nociceptin may be useful water diuretics for patients with water retaining diseases (Kapusta et al., 1997). As with the other opioids, nociceptin inhibits *in vitro* neurogenic contraction of the stomach and the small intestine in a variety of species, and it has been also shown that nociceptin stimulates food intake in satiated rats, therefore ORL_1 antagonists may possibly constitute new anorexic agents (Pomonis et al., 1996).
Having summarized the widespread sites of action the nociceptin and its receptor, the following section will be focused on the role of the nociceptin in the mechanisms of pain modulation.

3.1.2.2 Supraspinal nociceptin

In the first papers reporting the identification of nociceptin (Meunier et al., 1995; Reinscheid et al., 1995), it was shown that contrary to opioids, the i.c.v. injection of nociceptin has a pronociceptive dose-dependent effect, i.e. it reduces the tail flick and hot plate response latency in mice. Both groups interpreted these data as a hyperalgesic action; a decrease in nociceptive threshold produced by this peptide. Following these findings, several lines of studies obtained similar results (Calo et al., 2000), while few others disagreed since they observed both hyperalgesia and analgesic effects of supraspinal nociceptin (Rossi et al., 1997). Moreover, Mogil et al., (1996) documented an anti-analgesic or 'anti-opioid' activity of nociceptin by showing the direct antagonistic effect of nociceptin on systemic morphine analgesia and also knowing that many forms of stress-induced antinociception are mediated by endogenous opioids, they suggested that i.c.v. injection of nociceptin does not produce hyperalgesia as predicted previously but rather reverses opioid-mediated stress-induced antinociception.

This was very much a surprise since ORL_1 receptor and classical opioid receptors share the same transductional mechanisms. Although little is known on the mechanisms behind the supraspinal effect of nociceptin, it is reasonable to speculate that the opposite effect of nociceptin compared to the classical opioid receptors could be due to a distinct localisation of nociceptin and opioid peptides and their respective receptors on the neuronal networks involved in pain transmission.

In fact ORL_1 receptors and opioid receptors are not co-localized in nociceptive centres such as raphe nuclei, periaqueductal grey, amygdala, hypothalamic region (Schulz et al., 1996). Furthermore in the rostral ventomedial medulla (RVM) of the rat, nociceptin exerts a marked inhibitory action on all neurones (on-cells, off-cells and neutral-cells) and doing so blocks the activation of those cells by opioids (Heinricher et al., 1997). A similar effect was found when studying the actions of nociceptin within the PAG where nociceptin inhibited nearly all neurones present in

that area (Vaughan et al., 1997) and also attenuated the analgesic effect of locally injected morphine (Morgan et al., 1997). Overall, these studies suggest that the anti-analgesic effect of OFQ/N may be result of a generalized inhibition of all the descending analgesic pathways.

Thus these findings suggest that ORL_1 antagonists could provide a new class of supraspinally acting analgesics which could enable a reduction in morphine dosage, thus minimising the risk of tolerance and dependence since nociceptin does not possess abuse ability (Devine et al., 1996), but a confirmation of the role of nociceptin as an anti-opioid peptide will require the development of a specific ORL_1 receptor antagonist.

3.1.2.3 Peripheral nociceptin

In addition to effects in the CNS, nociceptin modulates nociception at the peripheral nervous system, again with somewhat conflicting results.

Work carried out in our laboratory shows that nociceptin at low doses (5 pmol to 1 nmol) administrated in the peripheral hind paw of anesthetised rats evokes excitatory responses from dorsal horn neurones (Carpenter et al., 2000). Similar responses were also observed by other groups showing the ability of peripheral nociceptin at very low doses (0.01 to 100 fmol) to produce a flexor response which is dependent upon release of substance P in peripheral nerve endings of primary nociceptive afferent neurones. The phenomenon can be blocked by NK1 receptor antagonists, depletion of substance P by capsaicin and is abolished in NK1 gene knockout mice (Inoue et al., 1998). However, the same group have also shown the ability of peripheral nociceptin at higher doses (> 100 fmol) to produce an analgesic effect, producing a complete block of substance P actions (Inoue et al., 1999).

Peripheral nociceptin seems to have effects opposite to what was seen at supraspinal levels where nociceptin action has been described as a functional inhibition of

analgesia (Heinricher et al., 1997; Morgan et al., 1997). This could suggest that the peripheral nociceptin may act through different mechanisms when compared to the supraspinal action or maybe there are several ORL_1 receptors and the peripheral receptor may be different from that in the CNS although nociceptin appears to be an agonist at both.

3.1.2.4 Spinal nociceptin

The presence of nociceptin and its receptor in the spinal cord, (Anton et al., 1996; Neal et al., 1999) suggest that this system may have a physiological role in the control of pain transmission at the spinal level. However, this role of nociceptin in modulating pain threshold in the spinal cord is also controversial. Since primary afferent neurones are bipolar cells, actions on the nociceptors of such neurones at the periphery could be expected to be similar to those at the spinal synapses (Inoue et al., 1999).

The effects of spinal NC, similar to those seen in the periphery, reveals a dose-dependent direction of effect: nociceptin at low doses (pmol) causes hyperalgesia (as evidenced by behavioural tests), and hypersensitivity to thermal and mechanical stimuli, while at higher doses (nmol) a number of laboratories have observed antinociceptive effects and blockade of the hyperalgesic effects produced by substance P (Mogil and Pasternak, 2001).

Although the mechanisms for the hyperalgesic or the facilitatory effect is not clear, nociceptin may have a similar effect to that of morphine, where low doses have been shown to increase the release of excitatory neurotransmitters from the spinal cord, possibly via a direct excitatory effect on sensory neurones (Crain and Shen, 1990). Additionally, it is generally believed that inhibitory synapses are blocked at a lower concentration of inhibitory agents than excitatory synapses (Wagman et al., 1967) and therefore disinibition of inhibitory neurones may generate a facilitatory effect within

a circuit from direct inhibitory agents such as morphine and nociceptin. On the other hand, studies have reported that i.t. injection of nociceptin even at higher doses produces hyperalsgesia/allodynia yet others have found no effect (Calo et al., 2000; Mogil and Pasternak, 2001). Despite many contradictions, most of the studies demonstrate that i.t. injection of nociceptin induces an antinociceptive effect similar to that evoked by classical opioid receptor agonists (Tian et al., 1997; Hao et al., 1998; Xu et al., 1999). This effect is further substantiated by electrophysiological findings showing a major inhibitory action of nociceptin on neuronal activity in the spinal dorsal horn (Stanfa et al., 1996). These observations underline the complexity of nociceptin probably due to the large distribution of its receptor and its ability to modulate both transmitter release and neuronal activity.

Acute pain rarely presents a clinical problem, therefore in order to conduct clinically relevant studies, its necessary to employ models involving more persistent pain including inflammatory and neuropathic related pain states. Since the efficacy and the potency of the analgesic effect of opioids are not the same in different pain conditions (as described in the section 3.1.1), in this thesis I will be investigating the role of nociceptin on spinal dorsal horn neurones in normal, nerve injury and sham operated animals with methods described in the section methods.

3.1.3 Oxycodone

Oxycodone is among the most commonly used opioid analgesics for the relief of postoperative pain, cancer pain and generally in chronic pain, for review see (Kalso, 2005). Indeed this effect of oxycodone is mainly due to its similarity to morphine and has been thought that oxycodone induces analgesia by acting on mu-opioid receptors (Beaver et al., 1978; Yoburn et al., 1995). However recent studies believe that the antinociceptive effect of oxycodone is mediated preferentially at kappa-opioid receptors, whereas morphine interacts primarily with mu-opioid receptors (Ross and Smith, 1997). From the literature it is well known that activation of mu-opioid receptors has been associated with the analgesic and most of the side effects

demonstrated by morphine (Kieffer, 2000). Therefore it was not surprising when several lines of evidence suggested that oxycodone evoked fewer side-effects than morphine (Kalso and Vainio, 1990; Maddocks et al., 1996; Mucci-LoRusso et al., 1998).

The mechanism of action of oxycodone is similar to other opioid agonists; briefly the common mechanism of opioids consist of a presynaptic activation of opioid receptor neurones whereby the release of neurotransmitters is reduced, a postsynaptic hyperpolarization of neurones which reduces the evoked activity in the neuronal pathway and disinibition of inhibitory neurones (Dickenson, 1994).

While oxycodone's analgesic effects are similar to those of morphine, other pharmacological characteristics distinguish these two opioids. Oxycodone has oral bioavailability of around 62% compared with morphine which is 23.9% (Hoskin et al., 1989; Leow et al., 1995) and systemic administration (s.c. or i.p.) of oxycodone produced a behavioural antinociceptive effect of about 2-4 times greater than morphine (Poyhia and Kalso, 1992). The high oral bioavailability of oxycodone maybe a result of its metabolite, oxymorphone, which has analgesic properties while morphine undergoes higher first pass metabolism and one of its main metabolites, morphine-3-glucuronide (M3G), has been suspected not to elicit any antinociceptive effect and/or antagonize the analgesic effect of morphine itself (Smith et al., 1990; Gong et al., 1992; Mucci-LoRusso et al., 1998).

In the thesis I will investigate the spinal effect of oxycodone using electrophysiological studies and also the antagonism of its action with naloxone and 1-[(3R, 4 R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2 benzimidazol-2-one known as J-113397 (the first non-peptide antagonist of nociceptin that binds to human and mouse brain ORL_1 receptors (Kawamoto et al., 1999) discussed further in chapter 5).

3.2 RESULTS

3.2.1 Nociceptin effects on evoked neuronal responses in normal animals

The effect of spinally applied nociceptin (50, 125 and 250 μg) was studied on n=6 (n= number of animals) dorsal horn neurones and each dose was followed for 60 min. The mean depth of neurones in this study was 723 ± 31 μm from the surface of the spinal cord which corresponds to lamina V-VI of the dorsal horn.

Analysis of all the neurones revealed that nociceptin had no significant effects with the first dose (50 μg) see Fig.2 and 3. However with 125 μg of nociceptin the following effects were observed: inhibition of C-fibre response of 62.13 ± 11% of control ($P< 0.01$), post-discharge 64.98 ± 24% ($P< 0.05$), input 69.67 ± 22% ($P< 0.05$) and less inhibition of the wind-up 44.03 ± 26% ($P<0.05$) see Fig.2. Furthermore nociceptin (125 μg) had no effect on innocuous responses mediated by Aβ-fibres and there was less inhibition of Aδ-fibre responses 41.68 ± 19% ($P< 0.01$), underlining the predominant effect of nociceptin in noxious evoked responses (see Fig.3). The inhibition was further increased with the highest dose (250 μg), suggesting a dose related response of this peptide see Fig.2-3 and Table2.

In fact, Fig.3A shows that the level of inhibition increased significantly on all the afferent fibres responses except the Aβ-fibre where this top dose of nociceptin did produce any changes.

These results are similar to several studies done in this laboratory (Stanfa et al., 1996; Carpenter et al., 2000), whereby the peptide can modulate nociceptive events by reducing wind-up, post-discharge and C-fibres of the neurones.

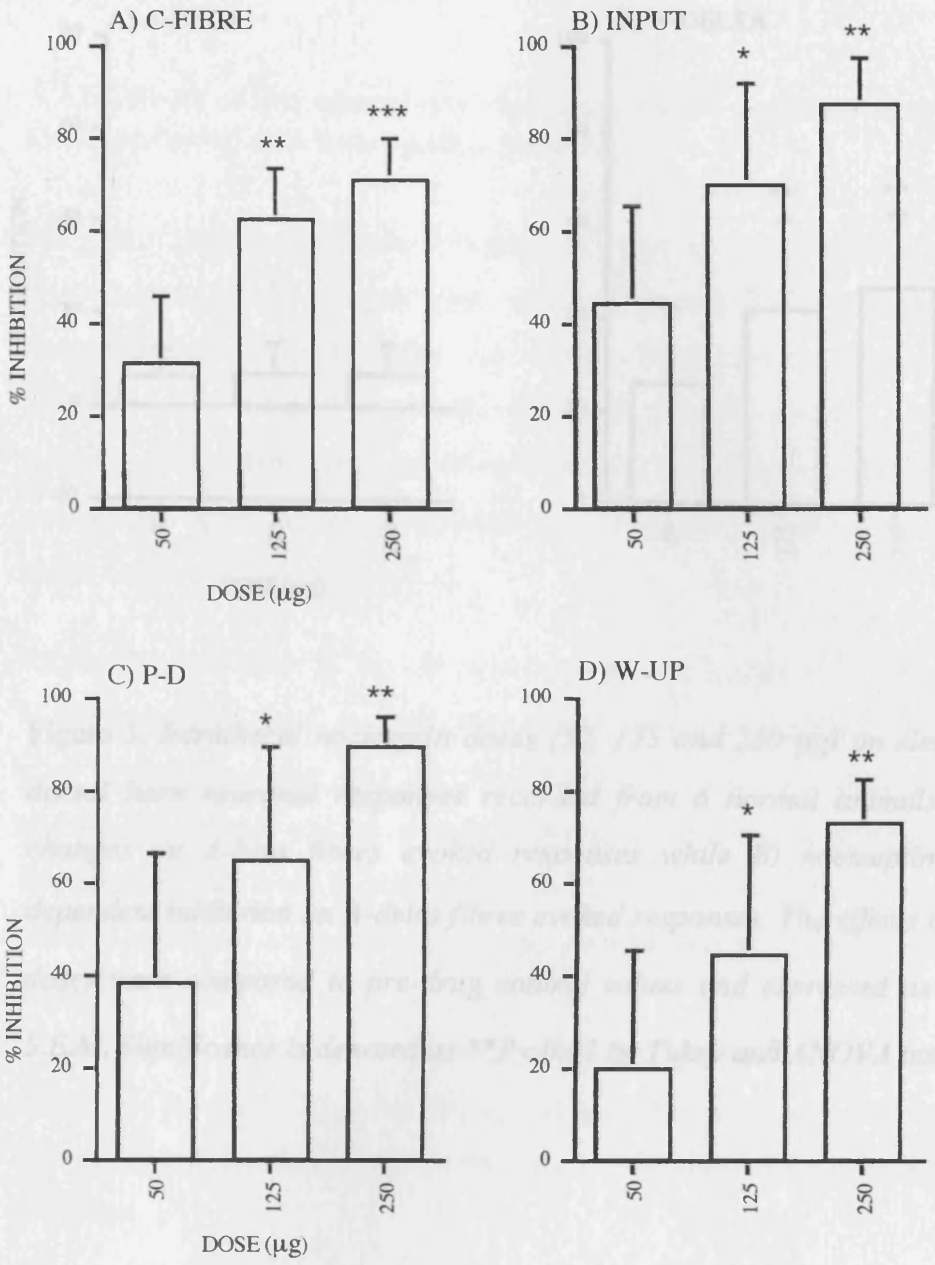

*Figure 2. Intrathecal nociceptin doses (50, 125 and 250 µg) causes dose-dependent inhibition on A) C-fibre, B) Input, C) Wind-up and D) Post-discharge evoked responses in normal animals (n=6). The effects of the individual doses were compared to pre-drug control values and expressed as % inhibition ± S.E.M. Significance is denoted as **P< 0.01, *P< 0.05 by Tukey and ANOVA tests.*

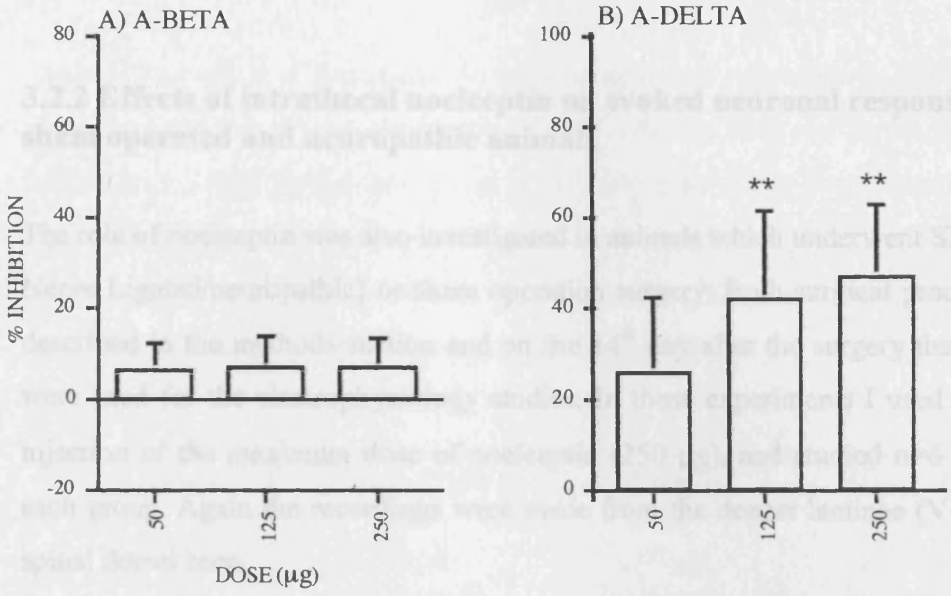

Figure 3. *Intrathecal nociceptin doses (50, 125 and 250 µg) on electrically evoked dorsal horn neuronal responses recorded from 6 normal animals, causes A) no changes on A-beta fibres evoked responses while B) nociceptin induces dose-dependent inhibition on A-delta fibres evoked responses. The effects of the individual doses were compared to pre-drug control values and expressed as % inhibition ± S.E.M. Significance is denoted as **P< 0.01 by Tukey and ANOVA tests.*

3.2.2 Effects of intrathecal nociceptin on evoked neuronal responses in sham operated and neuropathic animals

The role of nociceptin was also investigated in animals which underwent SNL (Spinal Nerve Ligated/neuropathic) or sham operation surgery. Both surgical procedures are described in the methods section and on the 14th day after the surgery these animals were used for the electrophysiology studies. In these experiments I used intrathecal injection of the maximum dose of nociceptin (250 μg), and studied n=6 animals in each group. Again the recordings were made from the deeper laminae (V-VI) of the spinal dorsal horn.

As presented in Table 2, the top dose of nociceptin had no significant effect on A-fibre evoked responses. In contrast nociceptin evoked a significant inhibition of C-fibre evoked responses and the post-discharge of the cells in both normal and neuropathic animals whereas this dose of nociceptin had no significant effect on C-fibre evoked responses in sham operated animals compared to baseline. Moreover wind-up and input response were significantly inhibited by 250 μg of nociceptin in normal animals.
In neuropathic animals these responses were also inhibited but not to the extent as that seen in normal animals. However, nociceptin had no effect on the input response in sham operated animals and causes a clear facilitation of the wind-up response in this group.

Overall it seems that the inhibition observed with 250 μg nociceptin is greater in normal animals, followed by the neuropathic animals and neurones in sham operated animals are the least effected by nociceptin (Table 2). In fact significant differences were found between sham operated rats compared to normal animals while overall no significance were found between normal and neuropathic animals (Table 2).
In addition to Tukey and ANOVA tests was employed Mann-Whitney test for the comparison of drug effects within the group and the level of significance was taken to be $P \leq 0.05$.

*Table.2. The effect of nociceptin on various neuronal responses in normal (n= 6), sham operated (n= 6) and neuropathic (n=6) animals. Data are expressed as the % inhibition ± S.E.M. Significant effects of the individual doses were compared to pre-drug control values of neurones within that group (**P< 0.01, *P< 0.05 by using Mann-Whitney test) and differences between groups (SNL or sham) were compared with normal animals ($^{+}$P< 0.05 using Tukey and ANOVA tests).*

Afferent fibres	***Normal rats with 250 μg Nociceptin % inhib. ± s.e.m.***	***Sham rats with 250 μg Nociceptin % inhib. ± s.e.m***	***SNL rats with 250 μg Nociceptin % inhib. ± s.e.m***
A-beta	6.94 ± 6.3	19.44 ± 5.9	2± 10.5
A-delta	46.6 ± 16.3	10.89 ± 11.3	26 ± 22.9
C-fibre	70.84 ± 8.9**	-3.75 ± 16^{+}	39.29 ± 4*
Post-discharge	89.42 ± 6.1**	-15.39 ± 10.5^{+}	65.68 ± 10.5*
Wind-up	72.88 ± 9.5**	-64.37± 9.1^{+}	69.3 ± 11.34
Input	87.02 ± 10.4**	12.9 ± 22.7^{+}	40.96 ± 22.8^{+}

3.2.3 Effects of oxycodone on evoked neuronal responses in normal animals

Oxycodone (1, 10, 50, 100 μg) was applied by intrathecal administration onto the spinal cord in normal animals (n=6) and each dose was followed over a time course of 60 min. Following the application of the highest dose of oxycodone, the effect of oxycodone was reversed by intrathecal administration of 50 μg naloxone on n=3 and the other half of the group was treated with 100 μg J-113397.

Intrathecal oxycodone produces a smaller inhibitory effect on the Aβ-fibre response when compared with that of the Aδ-fibre, C-fibre, post-discharge, input and wind-up responses. The lower doses of oxycodone (1 and 10 μg) induced very little inhibition of the Aβ-fibre response (Fig.4A). Even at the highest doses of oxycodone studied (50 and 100 μg) the maximum inhibition of Aβ-fibre response did not exceed 32 ± 4% (Fig.4A). Administration of naloxone revealed a reversal of oxycodone effects while J-113397 showed no reduction of the inhibition evoked by oxycodone. However these changes were not significant.

Oxycodone produces dose-dependent effects on Aδ-fibre evoked responses (Fig.4C). A modest facilitation was observed with the lowest dose (1 μg) followed by increases in inhibition as the dose applied is increased. For instance, the maximum percentage inhibition achieved was 76 ± 10% with 100 μg oxycodone ($P< 0.01$).

Similar dose-dependent effects of oxycodone were seen on the C-fibre evoked responses (Fig.4B), post-discharge and input of the neurones. Here again a peak inhibition of 63 ± 14% and 89 ± 5% was reached with the 100 μg of oxycodone on C-fibre ($P< 0.01$) and input ($P< 0.01$) evoked responses respectively (Fig 4B and 4D). In all cases the inhibition evoked by oxycodone was significantly reversed by naloxone, in contrast J-113397 failed to show significant changes.

The effect of oxycodone on the electrically evoked wind-up response was variable from one dose to the next. Oxycodone doses of 1, 10 and 50 μg all reveal facilitatory actions, with the greatest effect seen at 50 μg (19 ± 32%) However, at the maximum dose of 100 μg, a clear inhibition of 69 ± 10% was attained (data not shown). That inhibition was significantly reversed by naloxone and slightly reduced by J-113397.

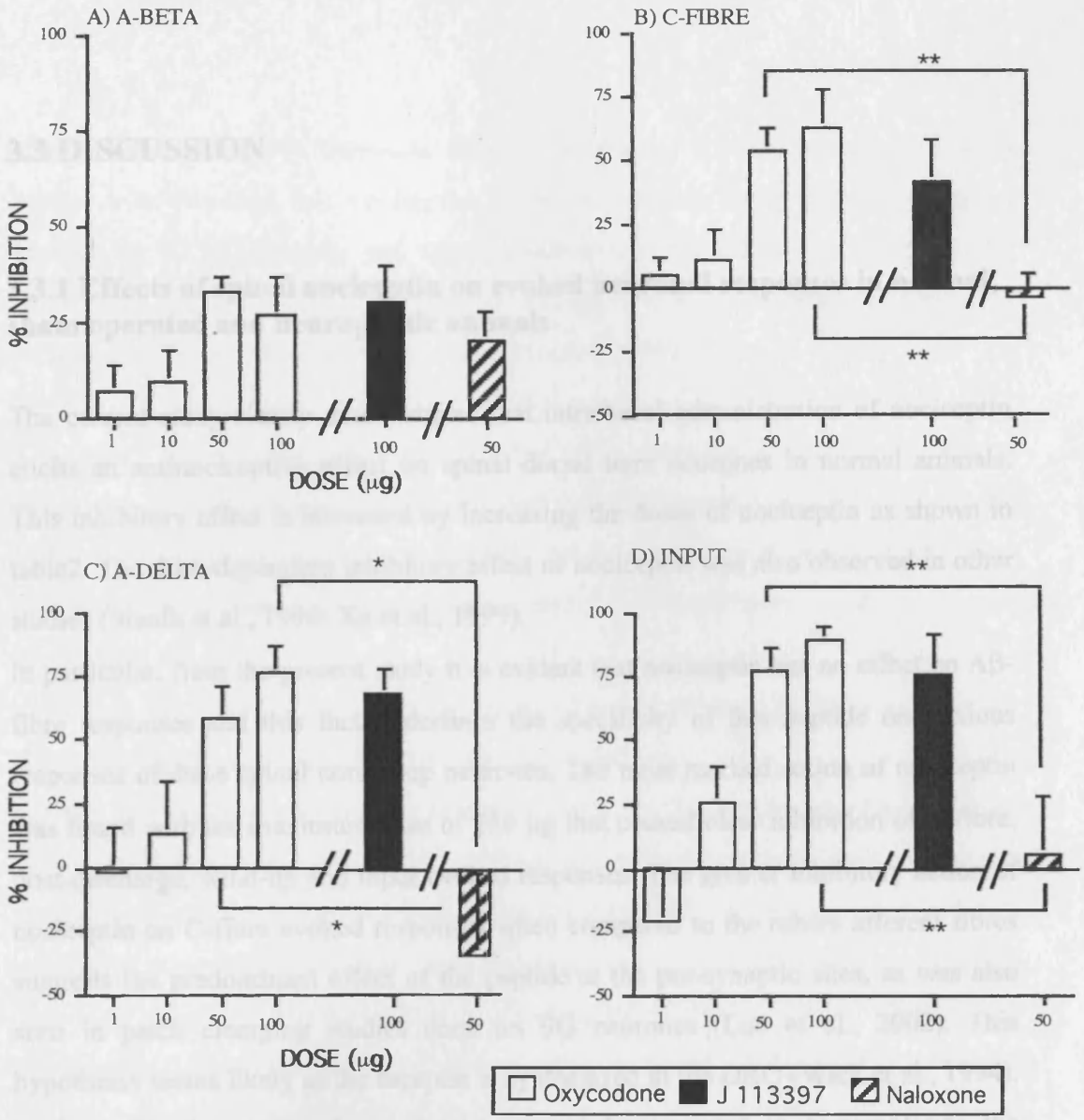

*Figure 4. The spinal effect of oxycodone (1, 10, 50 and 100 μg) induces: no change on A) A-beta evoked responses while on (B) C-fibre-, C) A-delta- and D) Input-evoked responses causes dose-dependent inhibition in normal animals (n= 6). After the application of oxycodone half of the animals were treated with spinal J-113397 (100 μg) and this did not reverse the inhibition seen with oxycodone, in contrast when the remaining animals were treated spinally with naloxone (50 μg), the inhibition induced by oxycodone was reversed. Data are expressed as the % inhibition ± S.E.M. Significance was found between the groups above indicated, **P<0.01 *P<0.05 by Tukey and ANOVA tests.*

3.3 DISCUSSION

3.3.1 Effects of spinal nociceptin on evoked neuronal responses in normal, sham operated and neuropathic animals

The current study clearly demonstrates that intrathecal administration of nociceptin elicits an antinociceptive effect on spinal dorsal horn neurones in normal animals. This inhibitory effect is increased by increasing the doses of nociceptin as shown in table2. The dose-dependent inhibitory effect of nociceptin was also observed in other studies (Stanfa et al., 1996; Xu et al., 1999).

In particular, from the present study it is evident that nociceptin has no effect on Aβ-fibre responses and this fact underlines the specificity of this peptide on noxious responses of these spinal cord deep neurones. The most marked action of nociceptin was found with the maximum dose of 250 μg that caused clear inhibition of C-fibre, post-discharge, wind-up and input evoked responses. The greater inhibitory action of nociceptin on C-fibre evoked responses when compared to the others afferent fibres suggests the predominant effect of the peptide at the pre-synaptic sites, as was also seen in patch clamping studies done on SG neurones (Luo et al., 2002). This hypothesis seems likely as the receptor is synthesized in the DRG (Wick et al., 1994). Actions directly on the deep neurones would be expected to hyperpolarize the neurone which would non-selectively reduce all responses. However, nociceptin acts also on the post-synaptic site by inhibiting the enhanced neuronal response elicited by repeated stimulation known as wind-up response. Also the post-discharge, another post-synaptic mechanism which underlies spinal facilitation was inhibited by nociceptin.

Both the pre- and post-synaptic effects of nociceptin are supported by the mechanism of action of this peptide in the spinal cord which is thought to act through three possible mechanisms. The first possible mechanism is the ability of nociceptin to inhibit voltage-dependent N-type Ca^{2+}channel currents (Connor et al., 1996) and therefore reduce the release of the afferent transmitters. The second is a postsynaptic

action where nociceptin increases inwardly rectifying K^+ conductance (Meunier, 1997). It is possible that nociceptin causes membrane hyperpolarization by an increase in K^+ conductance and this membrane hyperpolarization reduces cellular excitability and inhibits neurotransmitter release. The third possible mechanism is that nociceptin inhibits adenylate cyclase (Meunier, 1997).

The second part of these experiments studied the i.t. injection of a maximum dose of nociceptin in a group of neuropathic animals where the spinal nerves L5 and L6 were tightly ligated and a group of sham operated animals, whereby the surgical procedure was identical to that of the neuropathic group, but spinal nerve ligation was not performed.

Similar to the normal animals, this top dose of nociceptin has no effect on Aβ-fibre response in both sham operated and neuropathic animals.

Similar to the effects observed in normal animals, nociceptin produced a significant inhibition of C-fibre and post-discharge responses in neuropathic animals and overall the wind-up and input were reduced. Although the inhibitory response observed with nociceptin in normal animal was greater compared to neuropathic animals, nociceptin has maintained an analgesic role in neuropathic animals. Overall there is no significance difference in the effect of nociceptin in normal and neuropathic animals.

In fact behavioural studies reveal the effectiveness of nociceptin against thermal hyperalgesia resulting from either chronic constriction injury of the sciatic nerve (Yamamoto et al., 1997) or partial nerve injury in rats (Yamamoto and Nozaki-Taguchi, 1997). Abdulla and Smith, (1998) also found that axotomy increases the effectiveness of nociceptin on isolated dorsal root ganglion neurones.

However, there is limited information about the basis for the observed electrophysiological changes in the nociceptin system in the spinal cord following nerve injury and sham operated animals. Recent studies found, using the flexor reflex model, that nociceptin induces dose-dependent supression of the reflexes in this model in normal, acute inflamed and axotomized animals (Xu et al., 1999). The potency of nociceptin to inhibit the flexor reflex was greatest in animals with nerve

injury followed by normal animals with the inflamed animal group being the least effected (Xu et al., 1999). Interestingly the findings of Xu et al., (1999) are somewhat similar to what I have seen, namely, that the potency of nociceptin varied according to the following order: normal > injury > inflammation. The discrepancy between normal and nerve injury may be due to the neuropathic pain model used (Xu sectioned the entire sciatic nerve), the experimental measures (neurones vs reflexes).

However, others have reported a similar level of antinociceptive potency of nociceptin in normal and inflamed animals, while nociceptin had only a weak effect on the tail-flick latency of rats with peripheral nerve neuropathy (Hao et al., 1998). Also electrophysiological studies of spinal dorsal horn neurones demonstrate that i.t. effectiveness of nociceptin is increased in the presence of acute inflammation (Carpenter et al., 2000). In contrast, my experiments show that overall nociceptin has no inhibitory effect on sham operated animals, even though these animals persistent inflammation or persistent changes induced by an acute inflammation (post-operative period) may be suspected. One main difference is that the sham animals were used at 2 weeks whereas previous studies looked at acute effects of inflammation suggesting that the regulation of ORL_1 receptors may differ with time. Neuropathic animals also present sham effect as well as nerve injury while normal animals do not present any form of inflammation. Similar changes in sham operated animals were previously observed in the effect of another peptide, galanin (Flatters et al., 2002).

It is not clear why some changes are seen in sham animals at 2 weeks but recent studies suggested that following peripheral inflammation produced by s.c. injection of acute carrageenan there was a marked induction of the levels of nociceptin gene transcription in the DRG (Andoh et al., 1997). Nevertheless, this finding does not show a change in the density of ORL_1 receptors, which would be interesting as we are already applying high concentration of the peptide directly to the spinal cord. Also autoradiographical measures of nociceptin binding in the dorsal horn of the spinal cords from animals with chronic inflammation induced by peripheral injection of complete CFA showed that nociceptin binding increased in the superficial dorsal horn

(Jia et al., 1998). Although the densiometric analysis of autoradiograms showed nociceptin binding increased in medial and lateral laminae I–II with no change in binding occurring in lamina X (Jia et al., 1998), maybe this increase is due to the up-regulation of the peptide as also suggested above by Andoh et al., (1997) rather than increase of the number of receptors. These studies show once again the complexity of this receptor and further underlines the difference between this receptor and the other opioid receptors.

Moreover, the mechanisms underlying the reduced effect of nociceptin in neuropathic pain and the lack of effectiveness in sham operated animals needs to be further explored. However, Briscini et al., (2002) suggested an up-regulation of ORL_1 receptors in the spinal cord or dorsal root ganglia in animals with nerve injury.

Although, most studies predict an increase in nociceptin effects after inflammation and nerve injury, it is possible that in the two pathological conditions there is plasticity in peripheral and central nervous systems that could further enhance the response (or input) of the dorsal horn neurones more than in normal states so that inhibition is less as a result of higher levels of excitation. Thus this factor may therefore reduce the efficacy of nociceptin in these pain states. It seems from the finding of these experiments that peripheral inflammation and nerve injury may regulate endogenous nociceptin and its receptor in ways different from or opposite to the classical opioid system. Whether development of selective agonists or antagonists to the ORL_1 receptor will clarify the role of nociceptin and its receptor in pain transmission will be investigated in the next chapters.

3.3.2 Effects of spinal oxycodone on evoked neuronal responses in normal animals

The finding of this experiment indicates that oxycodone induces dose-dependent inhibition of spinal dorsal horn neurones. In particular there was clear inhibition of noxious induced responses when compared to non-noxious Aβ-fibre responses. This

is in agreement with the ability of opioids to produce a powerful inhibition of small afferent fibre input with little effect on large afferent evoked activity and led to speculation that opiates may predominantly act pre-synaptically (Yaksh and Noueihed, 1985). Alternately, the ability of opioids to suppress non-noxious activity in dorsal horn neurones by high doses suggested that the inhibition may be extended to postsynaptic neurones (Yaksh and Noueihed, 1985).

The inhibitory effect of oxycodone has been thought to be similar to that of morphine's and to be principally mediated through the mu-opioid receptor (Beaver et al., 1978; Yoburn et al., 1995). However, Ross and Smith, (1997) have suggested that the kappa-opioid receptor is responsible for the antinociceptive effect of oxycodone. They have based this hypothesis on the observation that the kappa-opioid receptor antagonist, norbinaltorphimine (nor-BNI), was able to attenuate the antinociceptive activity of oxycodone whilst sparing the antinociceptive effect of morphine.

The analgesic effect induced by oxycodone in our study was less than the effect of morphine obtained in similar studies (Suzuki et al., 1999). Indeed electrophysiological studies showed that the analgesic effect seen with i.t. morphine was more potent even though the concentration used was 10 times less than oxycodone (Suzuki et al., 1999). For example, Suzuki et al (1999) showed that i.t morphine (10 μg cumulative dose) produced about 85% of inhibition of C-fibre evoked response whereas in my study I found that oxycodone (100 μg cumulative dose) produced a 63% inhibition of the same afferent fibre evoked response. This reduced spinal effect of oxycodone was also seen when the antinociceptive effect was assessed on the tail-flick and hot plate tests after i.t. morphine (Plummer et al., 1990; Poyhia and Kalso, 1992), indicating that oxycodone has a poor affinity on spinal opioid receptors. This result maybe explained by the fact that the predominant opioid receptors in the spinal cord are mu and delta (Dickenson, 2001). On the other hand, the effect of oxycodone was almost similar to the inhibition observed with higher doses of nociceptin in normal animals. These could be summarized that i.t.

administration of those opioids agonists on dorsal horn neurones has a potency order as follows: morphine > oxycodone ≥ nociceptin.

My present findings have illustrated that after reaching significant antinociceptive effect with i.t. oxycodone (cumulative doses 1, 10, 50, 100 μg), the spinal effect of oxycodone was reversed with naloxone (50 μg) but not with J-113397 (100 μg). This is in agreement with the findings of (Poyhia and Kalso, 1992) where the antinociceptive effect of oxycodone was antagonized by naloxone. The situation is quite opposite with J-113397, where there is a total lack of antagonism. The only exception was the wind-up response where J-113397 seems to reduce the inhibitory effect of oxycodone, although all the other evoked responses were not reversed by the application of J-113397. Overall this result further underlines the selectivity of J-113397 (further details of J-113397 see chapter 5).

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CHAPTER 4

STUDIES WITH A NOCICEPTIN RECEPTOR AGONIST

4.1 INTRODUCTION

In the previous chapter the antinociceptive effect of nociceptin, the endogenous ligand of the opioid-like receptor 1 (ORL_1/NOP) was investigated in different pain states and raised the possibility that agonists at this receptor may represent a novel class of analgesics with therapeutic potential.

In an attempt to further understand the pharmacological characteristics and functional role of the ORL_1 receptor it was important to build on the identification of ORL_1 selective ligands. For instance, one of the first analogs of nociceptin was [Try^{14}]OFQ/N, developed to enable the detection of receptor binding and which has proven extremely valuable in the characterization of the receptor in both transfected cell lines and in the brain (Reinscheid et al., 1995). Another much studied analog of nociceptin is the peptide [Arg^{14}, Lys^{15}]NC, which has 3-fold greater binding affinity than nociceptin at human ORL_1 (Okada et al., 2000). Although this peptide has higher potency than nociceptin some unwanted effects such as ataxia were more pronounced and longer lasting than those elicited by nociceptin (Rizzi et al., 2002). Thus these effects may produce limitations in the behavioural assessment of the receptor function.

The lack of any nonpeptide agonist of ORL_1 receptor attracted the interest of several pharmaceutical industries in attempts to develop compounds as stable and bioavailable analogs of nociceptin.

In this chapter, for the first time, the *in vivo* electrophysiological effects of compound 20700857, a novel ORL_1 receptor ligand obtained from Solvay Pharmaceuticals will be investigated.

4.2 RESULTS

The effect of compound 20700857 was investigated on 36 dorsal horn neurones with mean depth 750 ± 50 μm from the surface of the spinal cord and this did not differ between groups; inflamed, normal and neuropathic rats. The compound was administrated intrathecally (i.t.) or subcutaneously (s.c.) at various doses (0.1, 1 and 10 mg/Kg) and each dose was followed for an hour.

In the carrageenan animals, the inflammation was induced by an injection of 2% carrageenan 100 μl in the plantar surface of the hindpaw of the rat (see methods for further details). I found that there were no changes in A-beta and C-fibre evoked responses in carrageenan and normal rats treated intrathecally or subcutaneously with 0.1, 1 and 10 mg/Kg of compound 20700857 when compared to their respective controls (see Fig.1)

However, post-discharge, input and wind-up of these rats showed some variations with respect to their controls (see Fig.2 and 3). In normal animals the lowest dose of i.t. compound 20700857 did not induce changes in the post-discharge response, while higher doses, 1 and 10 mg/Kg, evoked a slight inhibition of 27 ± 21% and 17 ± 34% respectively (See Fig.2). In contrast, s.c. injection of this compound caused a facilitatory effect on post-discharge response in the normal animals (see Fig.2). Greater effects of the drug on the post-discharge response was observed in carrageenan animals, where primarily i.t. and s.c. injection caused a facilitatory effect (see Fig.3). Moreover, after s.c. injection of 0.1 and 1 mg/Kg in normal animals the input response was facilitated by 12 ± 15%, while with the top dose of the compound 20700857, the input response was facilitated by 32 ± 14% (see Fig.2), while the i.t. injection of compound 20700857 in the same animal group produced a tendency to inhibition with the lower and the top dose, whereas 1 mg/Kg causes 7 ± 19% facilitation (See Fig.2). Again in carrageenan animals s.c. injection of the compound induces increased input responses, while the i.t. effect of the compound caused the opposite effect (see Fig.3).

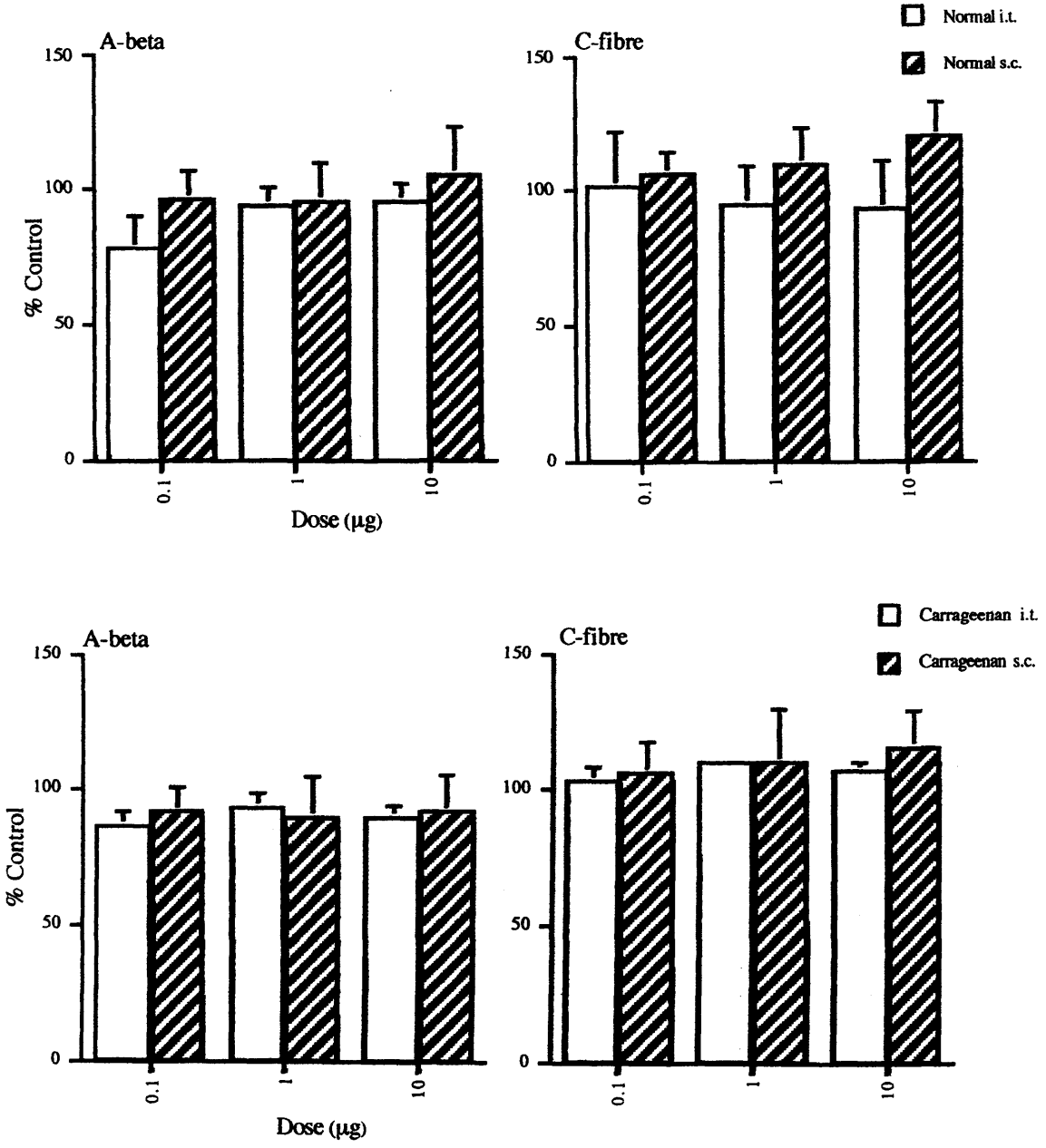

Figure 1. Intrathecal and subcutaneous administration of compound 20700857 has no effect on Aβ- and C-fibre evoked responses in normal (n=12) and carrageenan injected (n=12) animals. Data are expressed as % control ± S.E.M.

In carrageenan injected animals the wind-up response was increased with all three doses of i.t. compound 20700857, as well as the s.c. injection of the top dose (see Fig.3). The remaining doses, namely 0.1 and 1 mg/Kg of compound 20700857 administered subcutaneously induced a significant inhibition of the wind-up responses (53 ± 7% and 70 ± 11% respectively) of deep dorsal horn neurones (see Fig.3). Furthermore, subcutaneous injection of 10 mg/Kg of this compound caused significant inhibition of 38 ± 5% of the wind-up response in normal animals (Fig.2). Overall lower doses of s.c and i.t. injection of the compound induced slight inhibition of the wind-up response in normal animals (see Fig.2).

In addition to the electrophysiological study of carrageenan and normal animals, I also investigated the effect of i.t. and s.c. injection of compound 20700857 in neuropathic animals (see chapter 2 for description of the model), and the results observed were as follows:

The A-fibres responses were not altered significantly during i.t. or s.c. injection of the compound in study (see Fig.4). Furthermore, similar lack of change in response was found for C-fibre, input and post-discharge responses (Fig.5). Although in these experiments there was no significant difference between i.t and s.c. injected animals, overall i.t. administration of 10 mg/Kg compound 20700857 induces a slight inhibitory effect on the electrically evoked responses (Fig.4 and 5). On the other hand, mainly a facilitatory effect was seen following s.c administration of the compound 20700857 in neuropathic animals (Fig.4 and 5).

In neuropathic animals as previously seen in carrageenan injected animals, low doses of compound 20700857 appeared to have an inhibitory effect on the wind-up response (Fig.5), and the highest inhibitory action of compound 20700857 on wind-up response was reached 38 ± 27% after i.t. administration of 1 mg/Kg. However the inhibition observed was not significant. In contrast the s.c. injection of 1 and 10 mg/Kg of the compound in study induced significant increase of 147 ± 6% and 156 ± 6% on the wind-up response in neuropathic animal (Fig.5).

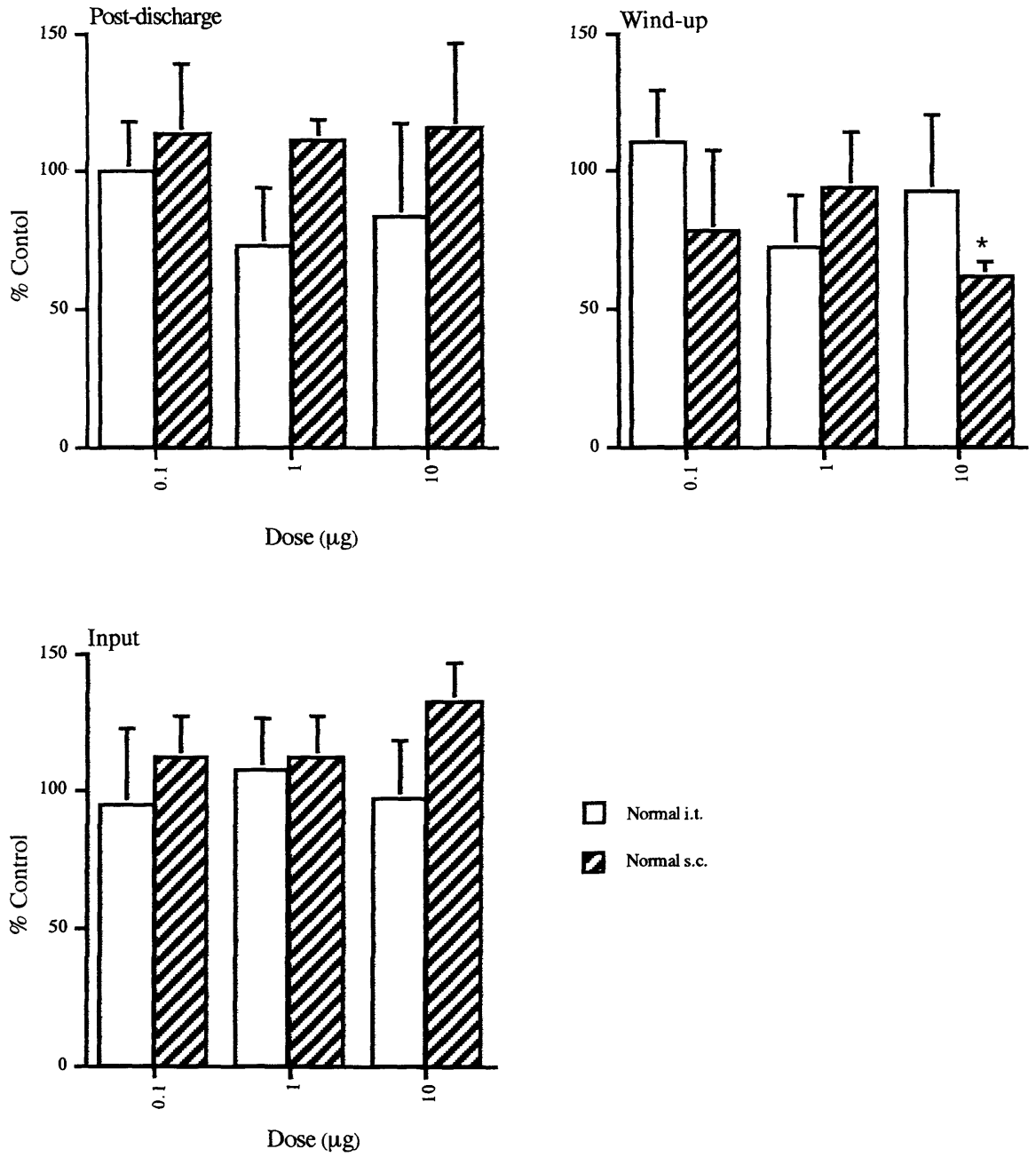

*Figure 2. Effect of intrathecally and subcutaneously applied compound 20700857 on post-discharge, wind-up and input responses in normal animals (n=12). The effects of the individual doses were compared to pre-drug control values and expressed as % control ± S.E.M. Significance is denoted as*P< 0.05 by Tukey and ANOVA tests.*

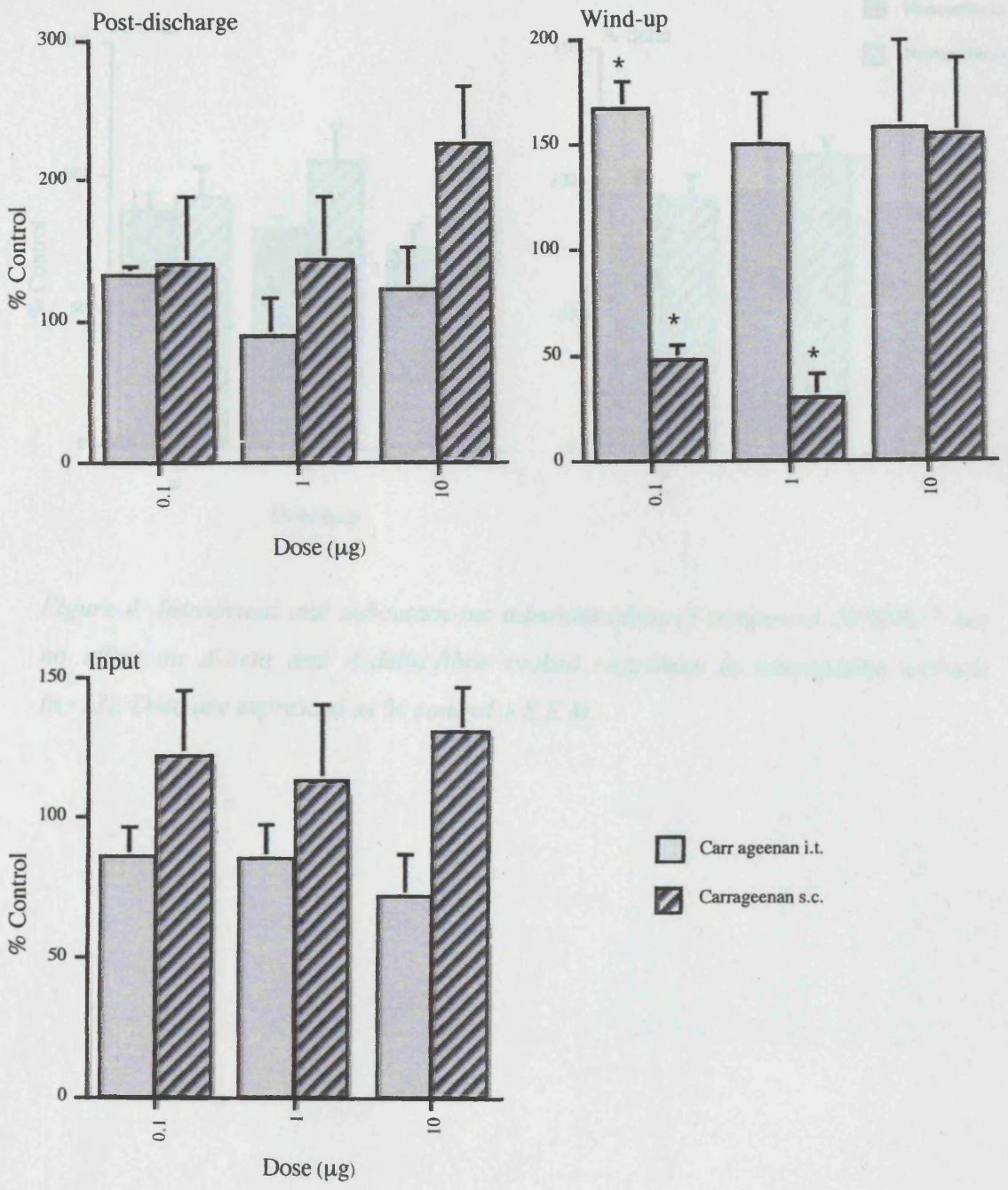

*Figure 3. Effect of intrathecally and subcutaneously applied compound 20700857 on post-discharge, wind-up and input responses in carregeenan injected animals (n=12). The effects of the individual doses were compared to pre-drug control values and expressed as % control ± S.E.M. Significance is denoted as*P< 0.05 by Tukey and ANOVA tests.*

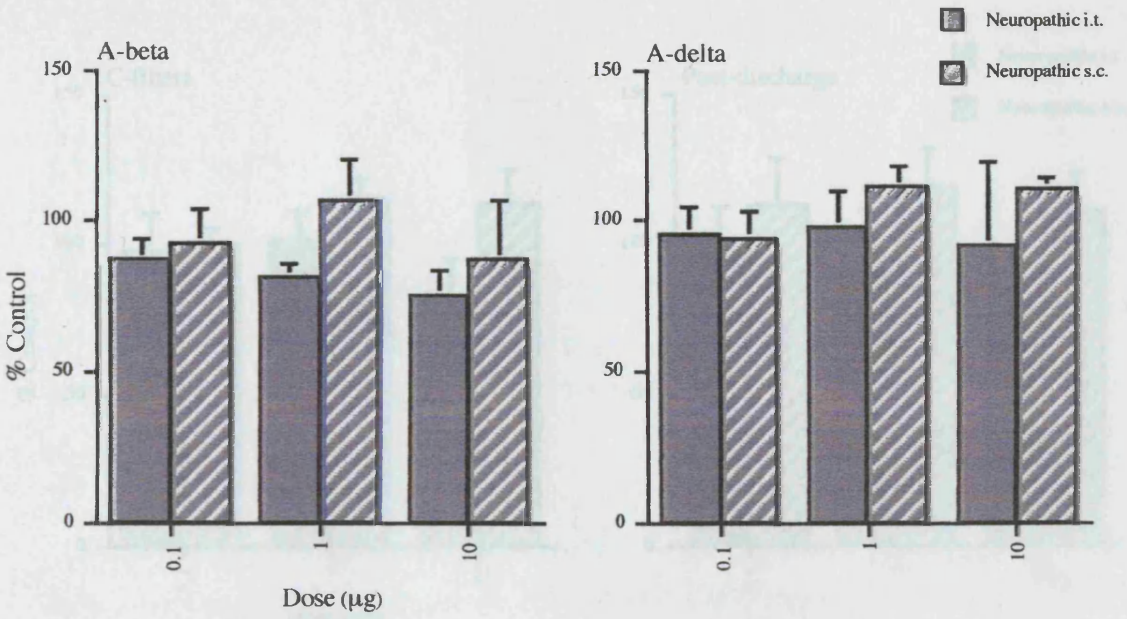

Figure 4. Intrathecal and subcutaneous administration of compound 20700857 has no effect on A-beta and A-delta-fibre evoked responses in neuropathic animals (n=12). Data are expressed as % control ± S.E.M.

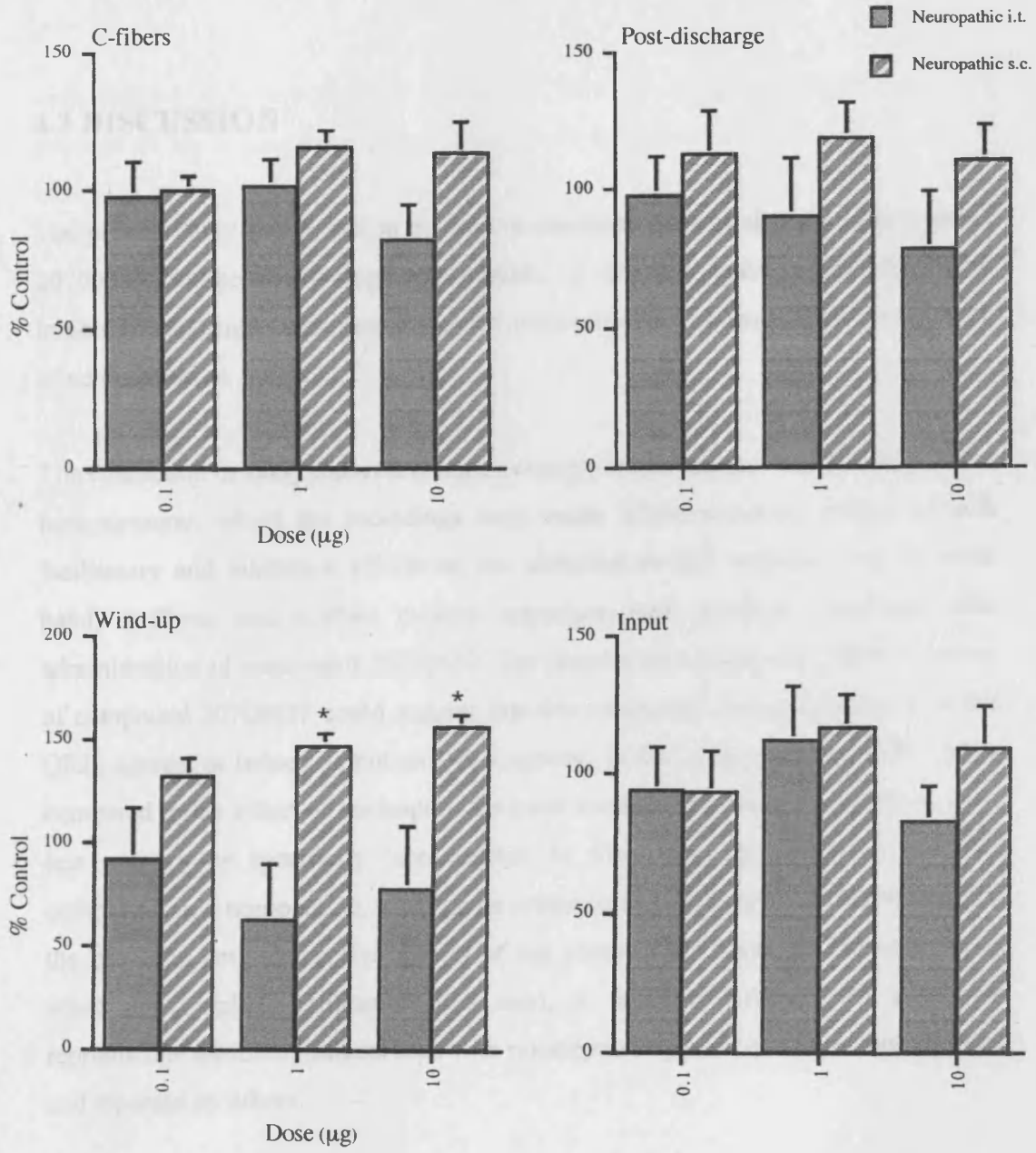

*Figure 5. Effect of intrathecally and subcutaneously applied compound 20700857 on C-fibre, post-discharge, wind-up and input responses in neuropathic animals (n=12). The effects of the individual doses were compared to pre-drug control values and expressed as % control ± S.E.M. Significance is denoted as*P< 0.05 by Tukey and ANOVA tests.*

4.3 DISCUSSION

The present study shows that in my *in vivo* electrophysiological study of compound 20700857, unlike the endogenous peptide, it did not cause a dose-dependent inhibition in normal, carrageenan injected and neuropathic animals whatever the route of administration.

The compound in study induced complex changes in the responses of the deep dorsal horn neurones where the recordings were made. These responses consist of both facilitatory and inhibitory effects on the electrical evoked response. On the other hand, A-fibres and C-fibre evoked responses were generally unaltered after administration of compound 20700857. The absence of a prominent inhibitory effect of compound 20700857 could suggest that this compound does not behave as a full ORL_1 agonist or indeed, is not an ORL_1 agonist. In fact, compound 20700857 when compared to the effect of nociceptin, the most studied ORL_1 agonists, seems to have less potency or specificity (see chapter 3). One other possibility is that this compound, as a non-peptide, when given systemically, has actions at sites where both the pro- and anti-nociceptive effects of the receptor are revealed. However, even when direct spinal application was used, it failed to produce the clear and reproducible inhibitory effects seen with nociceptin as presented in previous chapters and reported by others.

However, the overall results of these experiments shows that compound 20700857 has an interesting effect on the wind-up response and this varies with the rote of administration and the pain states (e.g. normal, carrageenan and neuropathic animals). In fact, the wind-up response was significantly increased by s.c. and i.t. administration of compound 20700857 in neuropathic and carrageenan injected animals respectively. By contrast, s.c. injection of this compound in normal and carrageenan animals induces inhibition of the wind-up responses vs their respective pre-drug control. This finding may well suggest that compound 20700857 has a role

in modulation of the mechanisms of wind-up.. Wind-up, as discussed in chapter 1 is a measure of the enhanced neuronal response elicited by repeated stimulation and is usually evoked by C-fibres stimulation leading to activation of NMDA receptors (Mendell and Wall, 1965; Dickenson and Sullivan, 1987). Since the present study shows that compound 20700857 did not cause significant changes on either the overall C-fibre evoked response or the input response (a measure of afferent excitability) both measures that are used for the calculation of the wind-up response, the data may suggest that compound 20700857 acts to modulate the NMDA receptor in a rather selective manner. However this effect and possible mechanisms needs to be further investigated.

In the present study the doses of compound 20700857 used were as advised by the company and they covered the range of doses that I have used previously, for instance the top dose was twice the concentration of nociceptin used in my electrophysiology study (chapter 3).

Overall the result of this study indicates that compound 20700857 is not as potent as nociceptin, the endogenous agonist of the ORL_1 receptor and subsequently the partial effect observed with the compound may be due to its ability to modulate other receptors that could include the NMDA receptor.

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CHAPTER 5

STUDIES WITH A NOCICEPTIN RECEPTOR ANTAGONIST

5.1 INTRODUCTION

One of the most common approaches to investigate the pharmacological activity of a certain receptor and to investigate the functional role of the ligand at the receptor is to find a selective antagonist of that receptor. In the case of nociceptin, there is general agreement that the spinal inhibitory effect of this peptide is not reversed by naloxone or requires high doses and even then is incomplete (Faber et al., 1996; Stanfa et al., 1996; Erb et al., 1997; Liebel et al., 1997; Hao et al., 1998). Thus, it appears that nociceptin-induced antinociception is mediated by a novel mechanism independent of classical opioid receptors. Subsequently several peptide and non-peptide antagonists have been identified to determine whether the effects of nociceptin might be mediated by ORL_1.

One of the most studied nociceptin antagonists is [$Phe^1\psi$(CH2-NH)Gly^2]-nociceptin (1-13)-NH_2. This peptide acts as a competitive antagonist in guinea big ileum and mouse vas deferens (Guerrini et al., 1998). The first study of [$Phe^1\psi$(CH2-NH)Gly^2]-nociceptin (1-1 3)-NH_2 using *in vivo* electrophysiological recordings of dorsal horn neurones found that the compound behaves as an agonist after spinal administration (Carpenter and Dickenson, 1998). The inhibitory effect of [$Phe^1\psi$(CH2-NH)Gly^2]-nociceptin (1-1 3)-NH_2 was more susceptible to naloxone reversal than that of nociceptin (Carpenter and Dickenson, 1998). This may suggest that [$Phe^1\psi$(CH2-NH)Gly^2]-nociceptin (1-1 3)-NH_2 has some activity on other central opioid receptors although it has been demonstrated to be selective for peripheral ORL_1 receptors (Guerrini et al., 1998). It was also demonstrated in behavioural studies that i.c.v and i.t injection of [$Phe^1\psi$(CH2-NH)Gly^2]-nociceptin (1-1 3)-NH_2 does not antagonise the effect of nociceptin on nociceptive transmission (Candeletti et al., 2000).

It is well known that peptide compounds generally can have the advantages of high selectivity and specificity and the disadvantages of metabolic instability and limited distribution after administration. Indeed recent researches have been directed to the

discovery of non-peptide compounds which show low selectivity and specificity but better pharmacokinetics (Calo et al., 2002).

The 1- [(3R, 4 R) -1-cyclooctylmethyl-3- hydroxymethyl-4- piperidyl] -3-ethyl-1, 3-dihydro-2 benzimidazol-2-one known as J-113397, is the first non-peptide antagonist of nociceptin and binds to the human and mouse brain ORL_1 receptor (Kawamoto et al., 1999). As a putative non-peptide antagonist, this seemed a potential candidate for determining the involvement of the nociceptin system in nociceptive transmission in an *in vivo* preparation.

To elucidate the antagonist effect of J-113397, the first *in vivo* electrophysiological study was made to determine the effects of spinally applied J-113397 after treatment with nociceptin on the electrical evoked responses of spinal dorsal horn neurones. Subsequently I investigated the role of the antagonist alone on electrical and naturally (mechanically) evoked responses in normal, carrageenan injected, neuropathic and sham operated animals. The aim was to ascertain if the antagonist could reveal any endogenous activity of nociceptin at the ORL_1 receptor in these different pain models.

5.2 RESULTS

5.2.1 Normal animals treated with nociceptin, J-113397 and morphine

In this experiment I used normal animals treated with 50, 125 and 250 μg of nociceptin as reported in section 3.2.1. Each dose was followed for 60 min and after the last dose of nociceptin, 100 μg of J-113397 was applied and followed for an hour. The effect of nociceptin could last at least 90-120 min (data not shown), and was therefore possible to see the effect of an antagonist by applying it 60 min after the spinal application of nociceptin. The study was done on n=6 dorsal horn neurones with a mean depth of 723 ± 31 μm.

The effect of nociceptin on the C-fibre evoked response of spinal neurones was reversed from 70.84 ± 9% inhibition to 19.77 ± 7% after 100 μg of J-113397 ($P< 0.01$), post-discharge from 89.42 ± 6% to 10.25 ± 16% ($P< 0.05$), wind-up from 72.88 ± 9% to +6.55 ± 19% ($P< 0.05$) and finally input from 87.02 ± 10% to 27.18 ± 12% ($P< 0.05$), see Fig.1A. Intrathecal J113397 reverses also the analgesic effect of nociceptin on A-delta but it could not be tested on A-beta evoked responses, since nociceptin had no effect on this response (Fig.2B).

Furthermore, to determine the selectivity of J-113397 versus ORL_1, I compared its action on another opioid receptor, with 1 μg of morphine being applied after J-113397 and I then followed the effect of the mu-opioid receptor agonist (n=4 neurones). This low dose of morphine produced clear inhibition of the C-fibre evoked responses, post discharge, input and windup even though J-113397 had completely reversed the action of nociceptin. For simplicity this effect is illustrated by the wind-up graph of a single dorsal horn neurones (Fig.2). Morphine produced a profound inhibition even in the presence of J-113397.

5.2.2 Normal and carrageenan animals treated with only J-113397

The effect of intrathecal injection of 100 μg J-113397 was studied on fourteen dorsal horn neurones; from normal rats (n=7) and in another group of rats 3 h after induction of carrageenan inflammation in the ipsilateral hindpaw (n=7). Their mean depth was 750 ± 50 μm from the surface of the spinal cord and this did not differ between groups. Electrically and naturally (von Frey) evoked neuronal responses were recorded from the two groups.

5.2.2.1 Electrically-evoked responses

The application of intrathecal J-113397 had no effect on the A-fibres responses of the carrageenan rats (Fig.3). J-113397, produced a slight facilitatory effect on Aδ- fibre responses in both normal (+18.3 ± 23%) carrageenan-injected (+21 ± 13%) Fig.3. Also in the post-discharge responses there was a tendency of J-113397 to cause a facilitation in the two groups of rats (Fig.3). The situation is quite different on C-fibre evoked responses where J-113397 had a mild inhibitory effect. This inhibition was greater in the carrageenan-injected rats (31 ± 12%) than in normal animals (17 ± 10%) see Fig.3. In addition wind-up was slightly inhibited in the carrageenan-injected rats but there was no effect in the normal rats whereas input responses were inhibited to a similar extent in both groups (Fig.4). Significance was not established on these electrically evoked responses when compared to their respective controls, thus overall 100 μg J-113397 was observed to have no significant effect on these neuronal responses in normal rats or those in the presence of inflammation. Thus, there is no evidence for an endogenous tone in the ORL_1 system under these conditions using electrical stimuli.

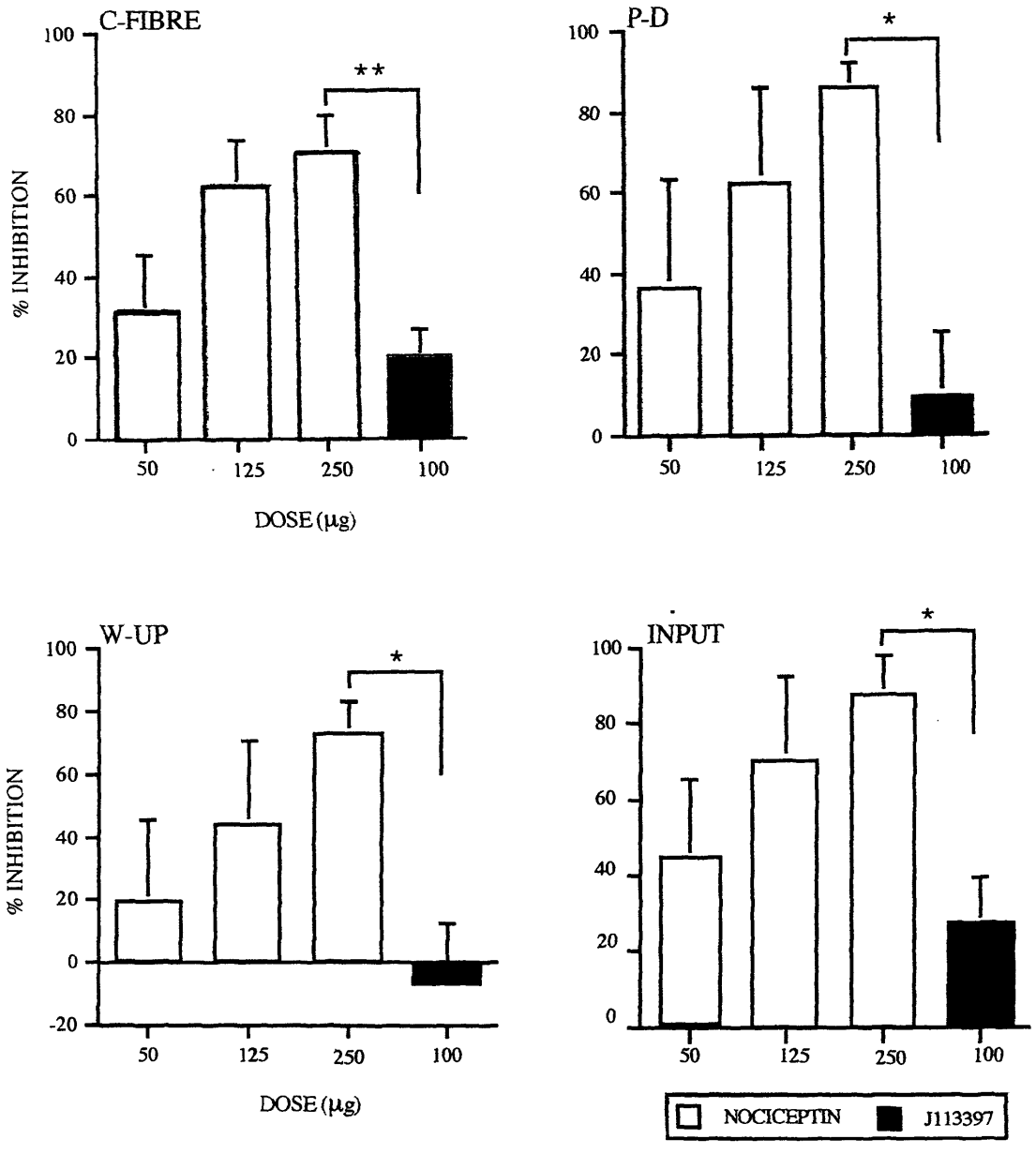

*Figure 1A. Intrathecal administration of nociceptin (50, 125, 250 μg) induces dose-dependent inhibition on C-fibre, post-discharge, wind-up and input evoked responses in normal animals (n=6). This inhibition was reversed by J-113397 (100 μg). Data are expressed as % inhibition ± S.E.M. Significance was found between the groups above indicated, *P<0.05 using Tukey and ANOVA tests.*

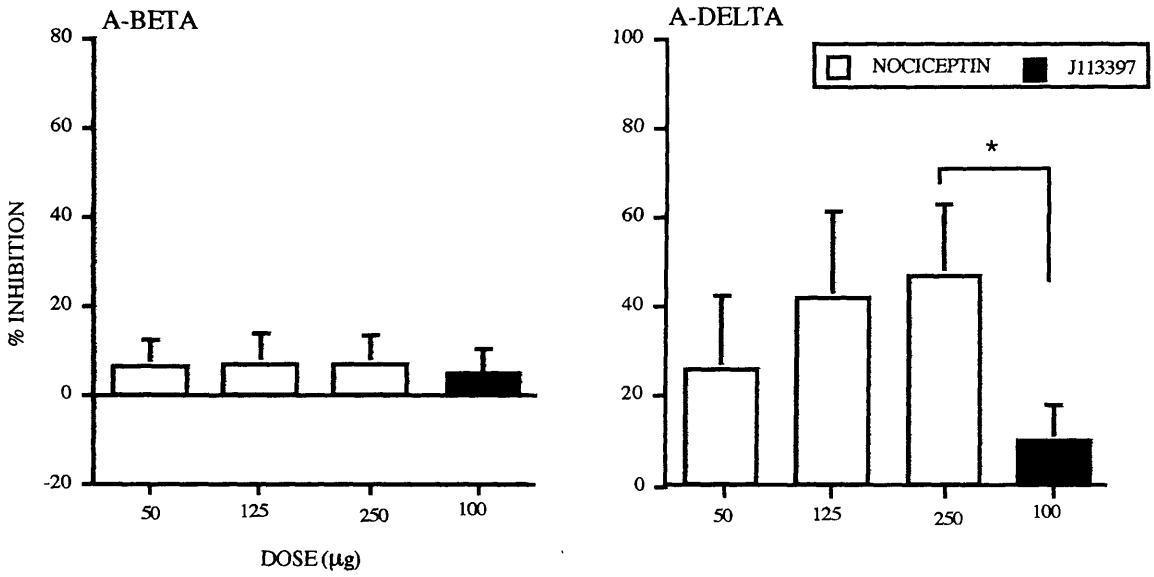

*Figure 1B. Intrathecal administration of J-113397 (100 μg) reverses the analgesic effect of nociceptin (50, 125, 250 μg) on A-delta evoked response but it could not be tested on A-beta evoked response, since nociceptin had no effect on this response (n=6 normal rats). Data are expressed as % inhibition ± S.E.M. Significance was found between the groups above indicated, *P<0.05 using Tukey and ANOVA tests.*

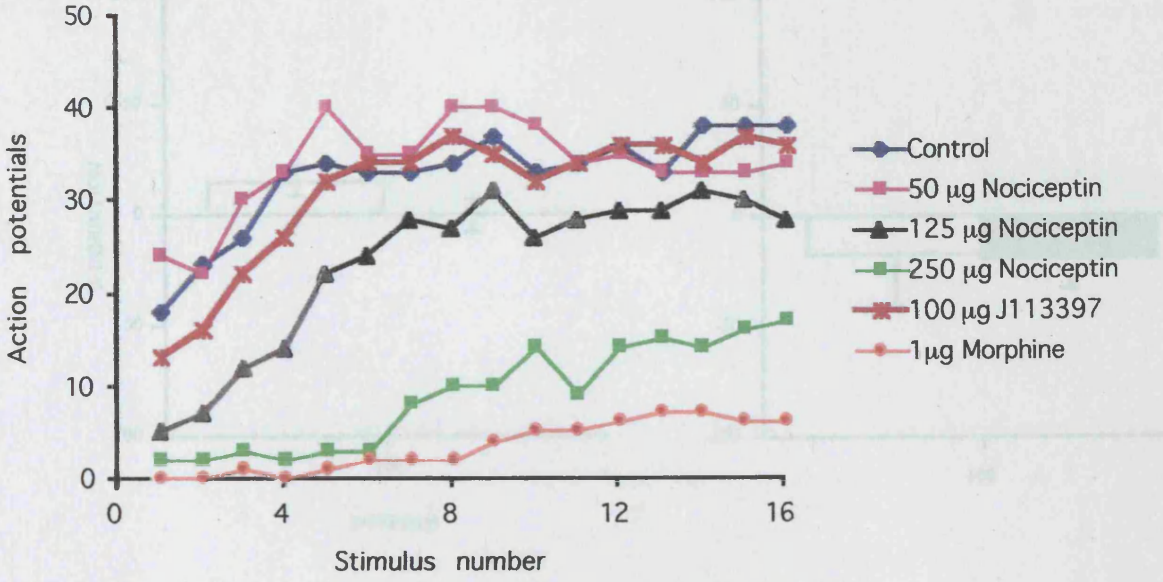

Figure 2. The wind-up response of a single dorsal horn neurone after intrathecal administration of nociceptin (50, 125, 250 μg), followed by J-113397 (100 μg) and then morphine (1 μg). The figure shows that nociceptin inhibits the wind-up in dose-dependent manner and this inhibition was reversed by J113397. J113397 did not prevent the inhibitory effect of morphine when applied on the same neurone.

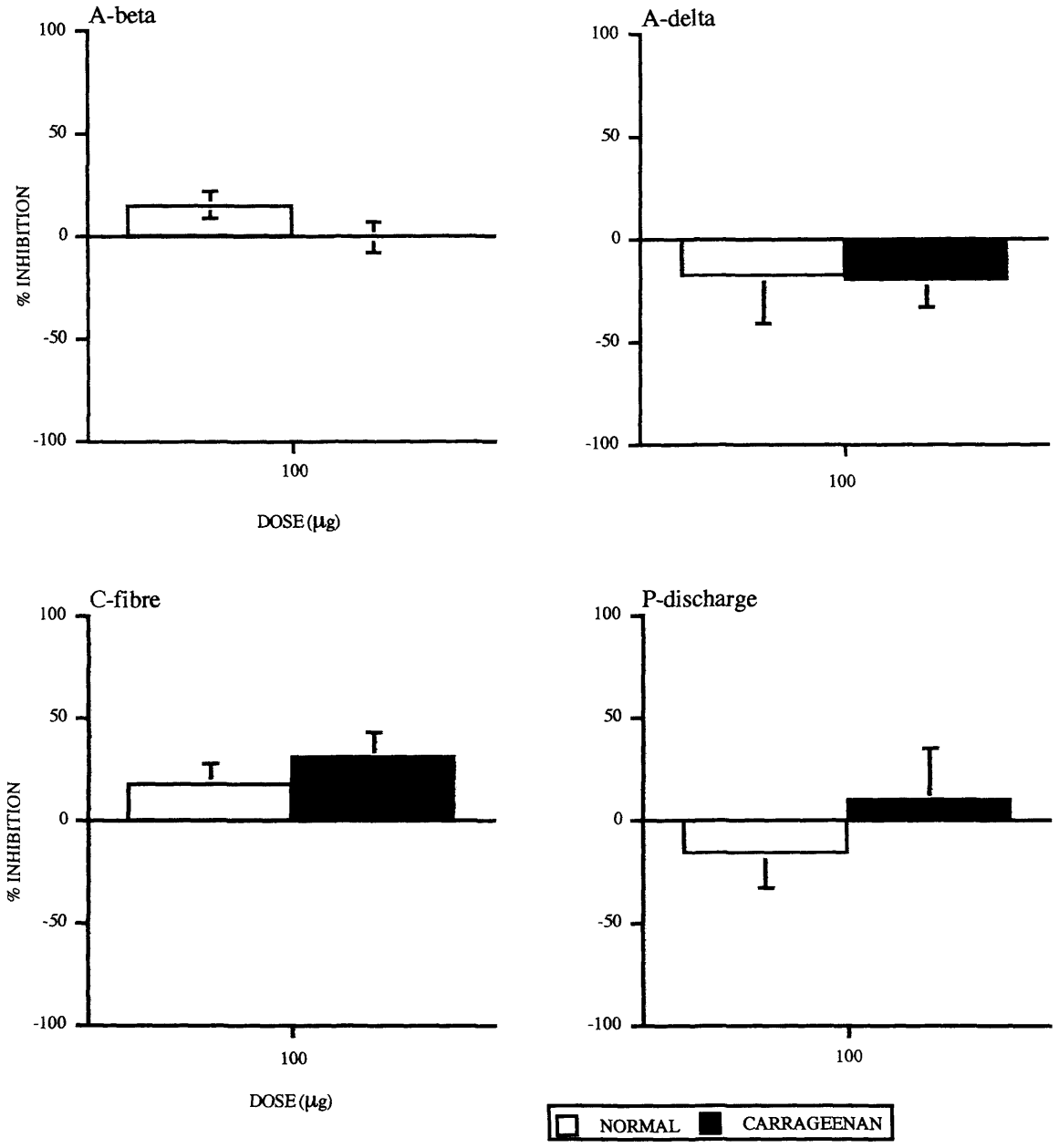

Figure 3. Spinally applied J-113397 (100 μg) has no effect on the Aβ-, Aδ-, C-fibre and post-discharge electrically evoked dorsal horn neuronal responses recorded from normal (n=7) and carrageenan (n=7) rats. Data are expressed as % inhibition ± S.E.M.

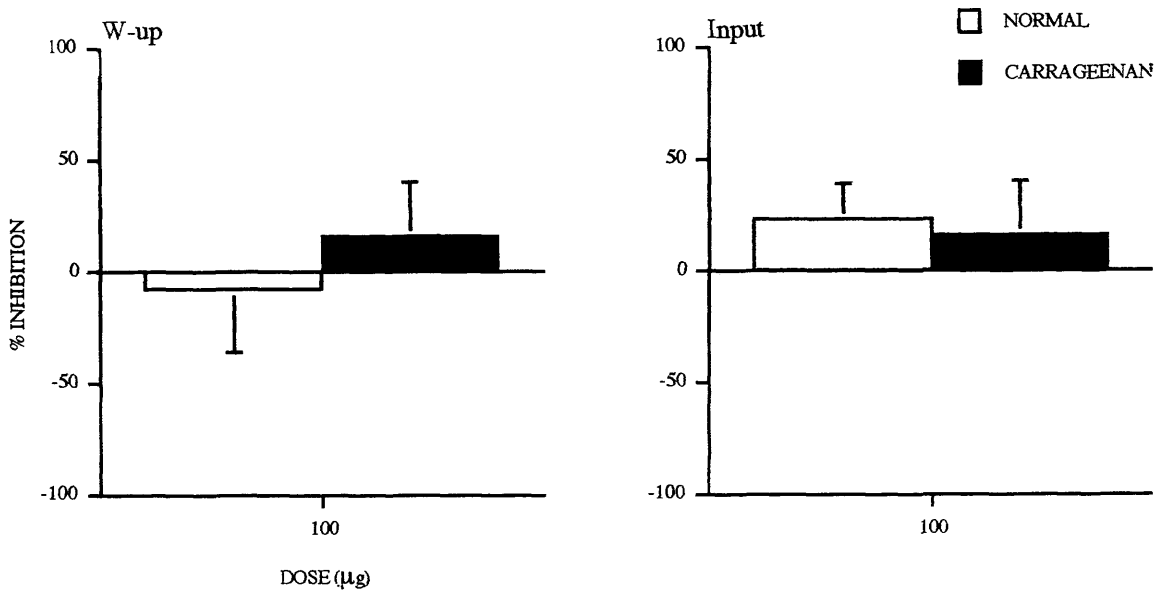

Figure 4. Spinally applied J-113397 (100 µg) has no effect on the wind-up and input electrically evoked dorsal horn neuronal responses recorded from normal (n=7) and carrageenan (n=7) rats. Data are expressed as % inhibition ± S.E.M.

5.2.2.2 von Frey evoked responses

To further identify the role of J-113397 on noxious and innocuous responses, I investigated the effect of J-113397 on mechanical evoked responses by using von-Frey filaments. The use of von Frey forces produced progressive increases in the neuronal firing rate proportional to the increase in the von Frey filaments. However, there were no changes in von-Frey response in normal animals after J-113397 injection. Similar responses were also observed in animals with carrageenan induced inflammation. Interestingly, in these animals with peripheral inflammation, the coding of these mechanical stimuli by the spinal neurones was increased compared to normal animals for both the low intensity and noxious forces, indicative of hypersensitivity in spinal processing.

This study clearly demonstrates that the 100 µg of J-113397 compound had no significant effect in both normal and inflamed rats. The number of action potentials at each von Frey was higher in the carrageenan-injected groups from the control

situation when compared to the normal animals and this difference remains the same also after the injection of J-113397. The highest von Frey (75 g) produced a response of 697 ± 191 action potentials in carrageenan animals before the injection of J-113397 and after the injection the response was 656 ± 201 action potentials, while the same von Frey produces 356 ± 73 in normal animals without J-113397 and 314 ± 68 action potentials after J-113397-injection (Fig.5). This underlines that there is difference in baseline responses between animals with inflammation and normal animal, yet J-113397 has no significant effect on these animals when compared to their respective control values.

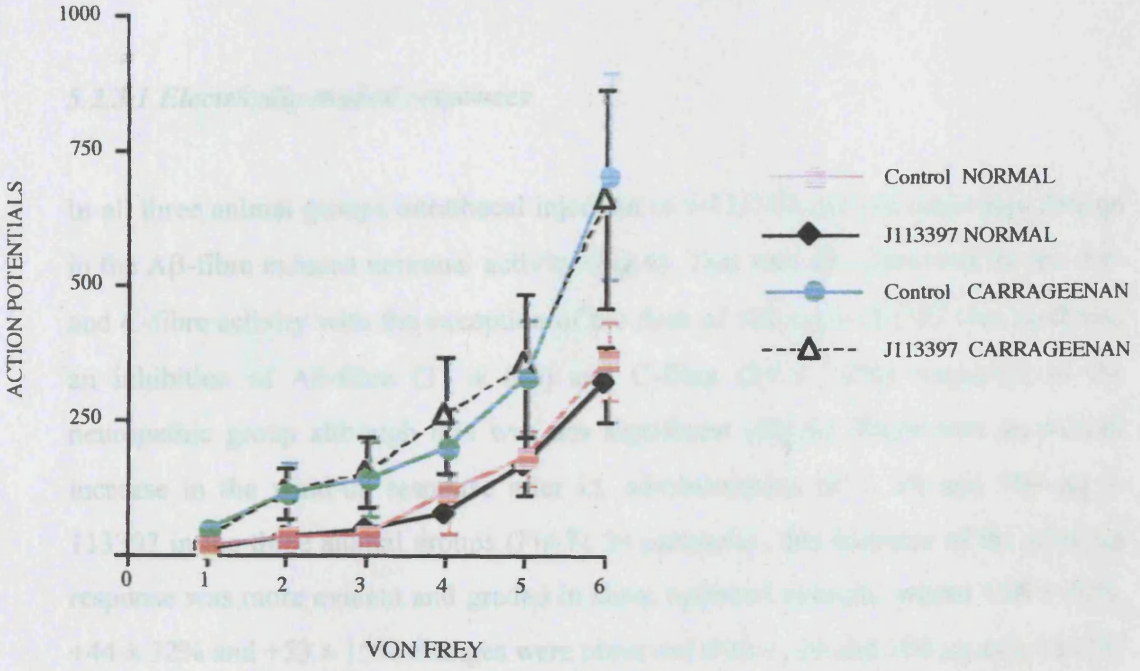

Figure 5. Effect of spinally applied J-113397 (100 μg) on the naturally evoked dorsal horn neuronal responses recorded from normal (n=7) and carrageenan (n=7) rats. The control values were obtained before drug administration. The von-Frey filaments 1-6 correspond to weights 2, 5, 9, 15, 30, 75g respectively. Data were expressed as mean number of action potentials evoked over 10 sec ± S.E.M.

5.2.3 Neuropathic and sham operated animals treated with only J-113397

The effect of intrathecal injection of J-113397 in cumulative doses (1, 10, 100 μg) was studied on twelve dorsal horn neurones; sham-operated (n=6) and neuropathic (n=6) rats. Their mean depth was 750 ± 50 μm from the surface of the spinal cord and this did not differ between groups. Electrically and naturally (von Frey) evoked responses were recorded from spinal dorsal horn neurones in each of the two groups and was compared with data of the normal animals obtained from the section 5.2.2.

5.2.3.1 Electrically-evoked responses

In all three animal groups intrathecal injection of J-113397 did not cause any change in the Aβ-fibre induced neuronal activity (Fig.6). This was also observed for the Aδ- and C-fibre activity with the exception of the dose of 100 μg J-113397 that produced an inhibition of Aδ-fibre (37 ± 8%) and C-fibre (29 ± 12%) responses in the neuropathic group although this was not significant (Fig.6). There was an overall increase in the wind-up response after i.t. administration of 1, 10 and 100 μg J-113397 in the three animal groups (Fig.7). In particular, this increase of the wind-up response was more evident and graded in sham operated animals, where +18 ± 20%, +44 ± 32% and +53 ± 15% changes were observed with 1, 10 and 100 μg of J-113397 respectively. However this facilitation was not considered significant when compared to the respective control values. In all three animal groups the input and post-discharge responses showed little change except for the input in the neuropathic rats at the highest dose where there was 41 ± 13% inhibition but again significance could not be established (Fig.7). Hence overall, 1, 10 and 100 μg J-113397 had no significant effect on the electrically-evoked neuronal responses of normal, sham or neuropathic rats.

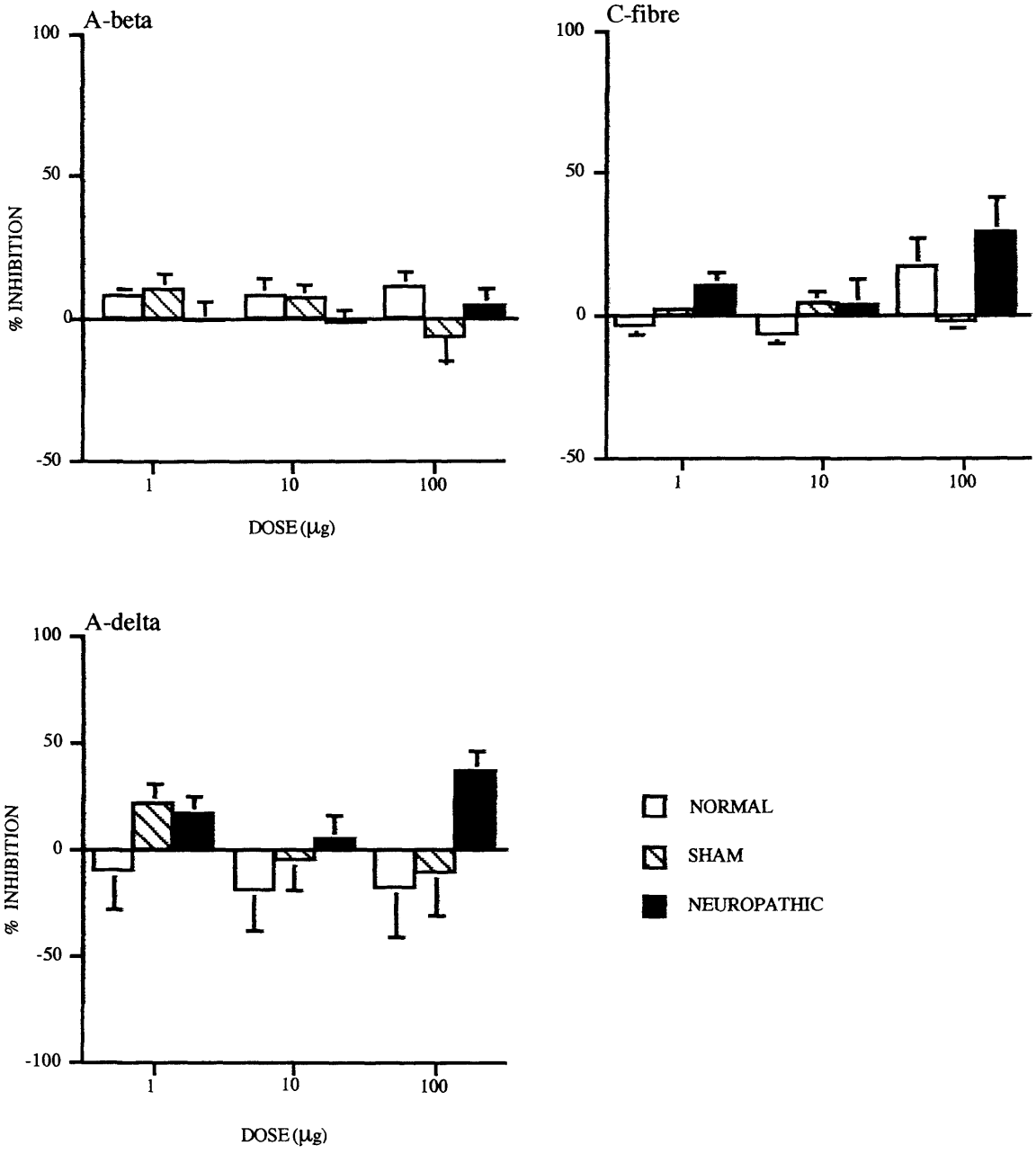

Figure 6. Effect of cumulative spinal administration of J-113397 (1, 10, 100 μg) on the Aβ-, Aδ- and C-fibres electrically evoked dorsal horn neuronal responses recorded from normal (n=7), sham(n=6) and neuropathic(n=6) rats. Data were expressed as % inhibition ± S.E.M.

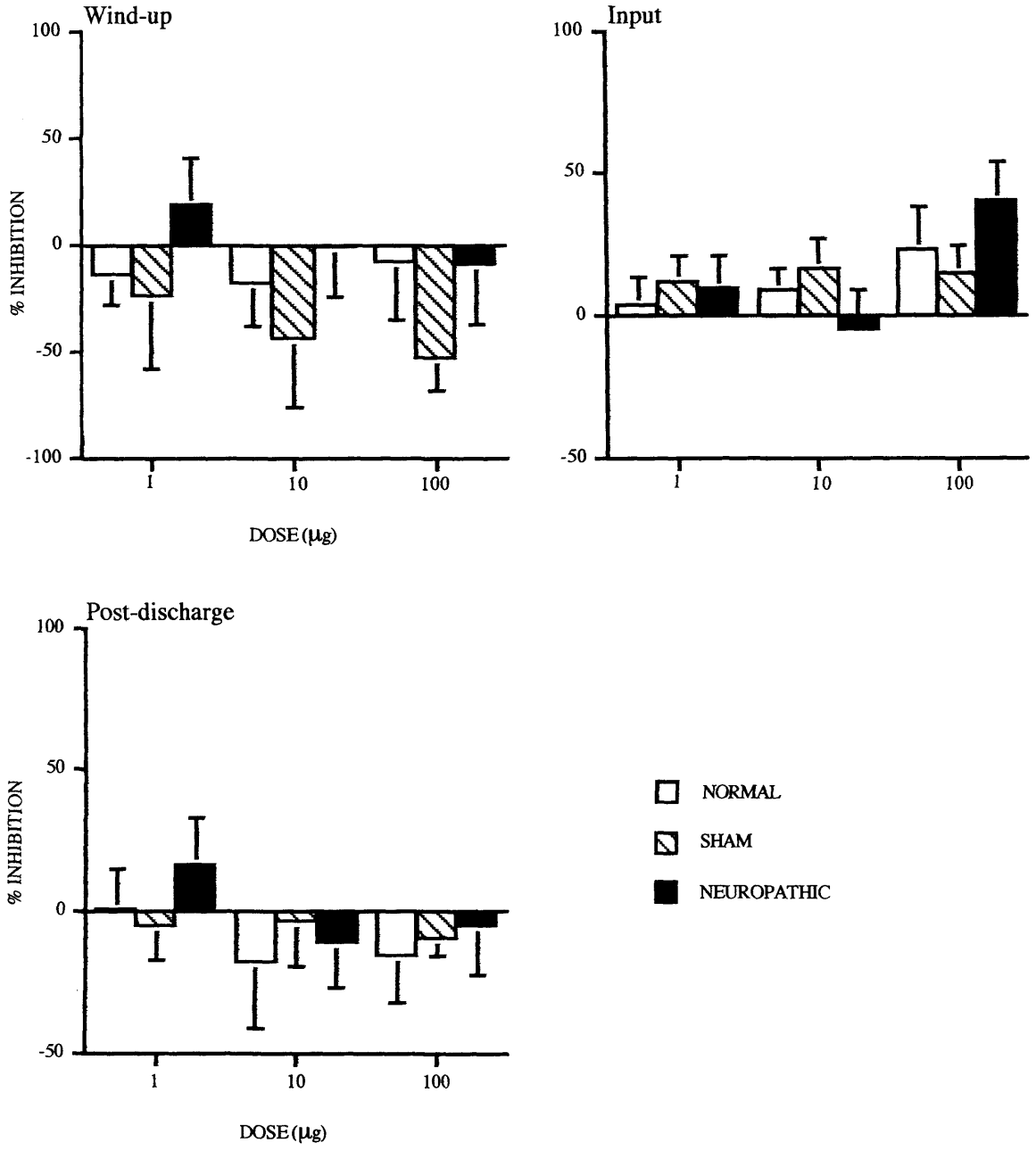

Figure 7. Effect of cumulative spinal administration of J-113397 (1, 10, 100 μg) on the wind-up, input and post-discharge electrically evoked dorsal horn neuronal responses recorded from normal (n=7), sham (n=6) and neuropathic (n=6) rats. Data were expressed as % inhibition ± S.E.M.

5.2.3.2 von-Frey evoked responses

Neurones in normal and sham animals injected with the lower doses (1, 10 μg) of J-113397 responded with no overall change compared to their control values (Fig.8 A and B). In the presence of neuropathy the neuronal responses were slightly inhibited by these lower doses compared to controls but this change was not significant (Fig.9A). The neurones were generally more responsive in the neuropathic and sham operated rats reflected in the higher number of action potentials produced by the mechanical stimuli (Fig.10).

There was no difference observed in the effects of J-113397 100 μg between neuropathic and sham groups and for clarity the results from the normal group are not displayed on the graph (Fig.9B). The sham and neuropathic rats injected with J-113397 100 μg showed less response to the various von Frey stimuli than their individual controls although significance could not be established. Overall the neuropathic rats injected with J-113397 had reduced responses compared to the J-113397-injected sham rats but there was no significant difference.

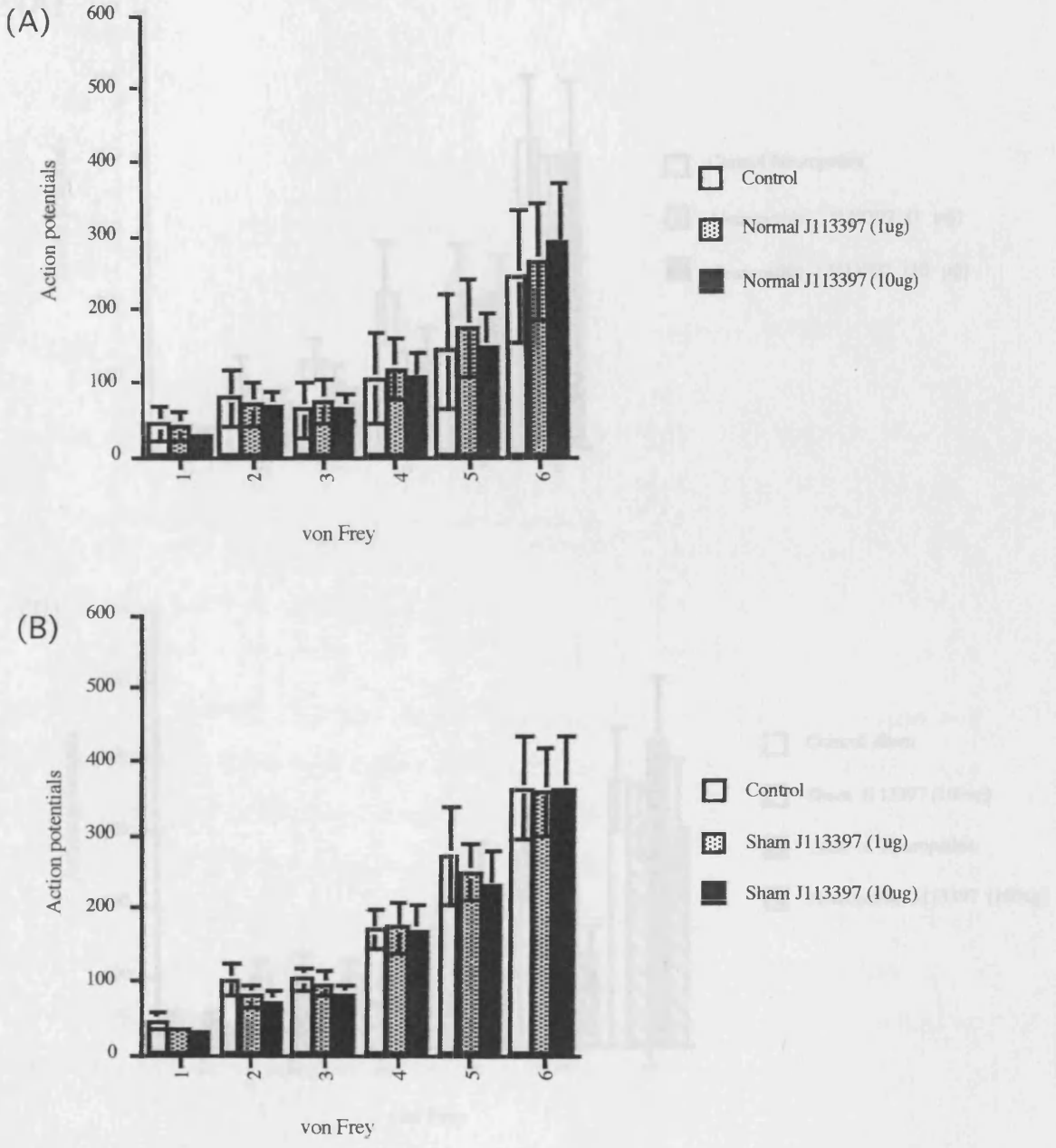

Figure 8. The figure shows that intrathecal application of J-113397 (1 and 10 μg) did not produce changes on the von Frey response in: (A) normal (n=7) and (B) sham operated (n=7) animals. The control values were obtained before drug administration. The von Frey filaments 1-6 correspond to weights 2, 5, 9, 15, 30 and 75 g respectively. Data were expressed as mean number of action potentials evoked over 10 sec ± S.E.M.

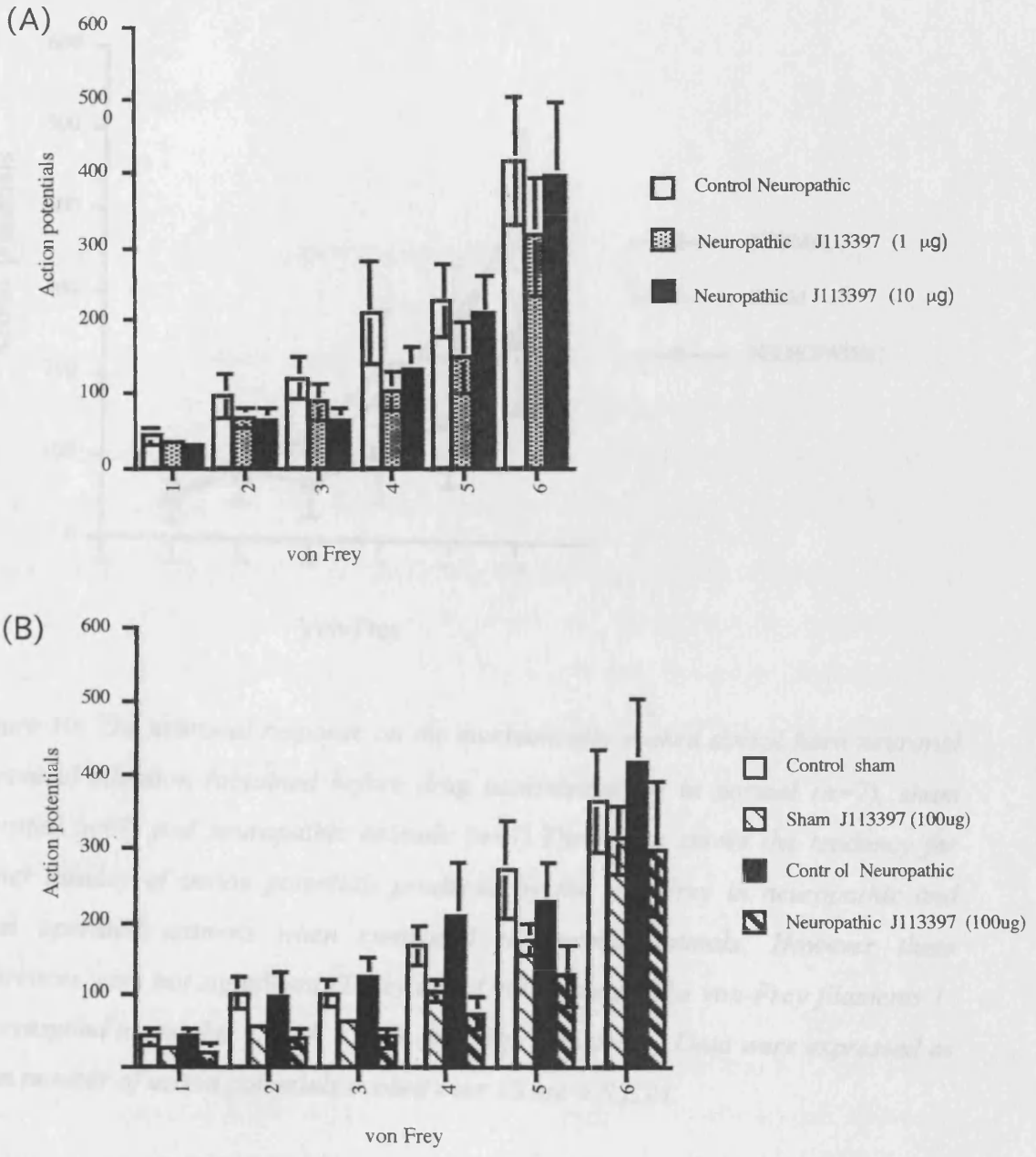

Figure 9. The figure shows that: A) spinally applied J-113397 (1 and 10 µg) has no effect on the von Frey response in neuropathic animals (n=6). Also in (B) the spinal application of J-113397 (100 µg) did not produce a significant difference for the von Frey response in sham (n=7) and neuropathic (n=6) animals. The control values were obtained before drug administration. The von Frey filaments 1-6 correspond to weights 2, 5, 9, 15, 30 and 75 g respectively. Data were expressed as mean number of action potentials evoked over 10 sec ± S.E.M.

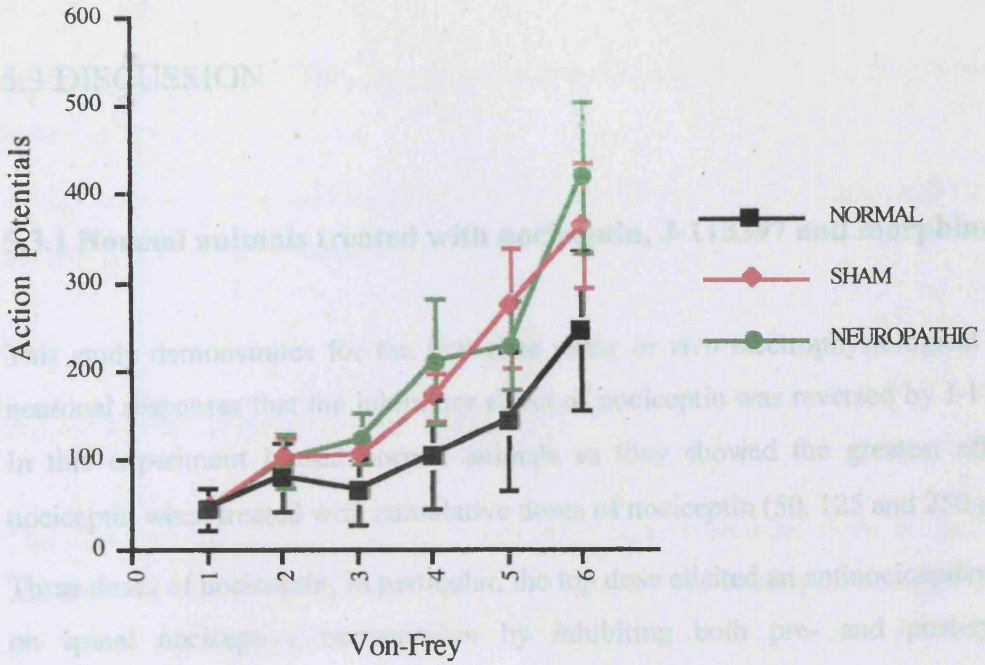

Figure 10: The neuronal response on the mechanically evoked dorsal horn neuronal in control situation (obtained before drug administration) in normal (n=7), sham operated (n=7) and neuropathic animals (n=7).The figure shows the tendency for higher number of action potentials produced by the von Frey in neuropathic and sham operated animals when compared to normal animals. However these differences were not significant (Tukey and ANOVA tests) .The von-Frey filaments 1-6 correspond to weights 2, 5, 9, 15, 30 and 75g respectively. Data were expressed as mean number of action potentials evoked over 10 sec ± S.E.M.

5.3 DISCUSSION

5.3.1 Normal animals treated with nociceptin, J-113397 and morphine

This study demonstrates for the first time using *in vivo* electrophysiological spinal neuronal responses that the inhibitory effect of nociceptin was reversed by J-113397. In this experiment I used normal animals as they showed the greatest effect of nociceptin when treated with cumulative doses of nociceptin (50, 125 and 250 μg).

These doses of nociceptin, in particular, the top dose elicited an antinociceptive effect on spinal nociceptive transmission by inhibiting both pre- and post-synaptic responses such as A-delta fibre, C-fibre, input, wind-up and post-discharge of the recorded neurones. In all studies I used J-113397 after obtaining the maximum inhibition with nociceptin and clear and almost complete reversals were observed. This data is in agreement with the findings on the opposite effect of nociceptin in the brain where s.c. injection of J-1133397 causes a dose-dependent inhibition of the hyperalgesia elicited by i.c.v. nociceptin in a tail-flick test with mice (Ozaki et al., 2000). Furthermore, *in vitro* studies of mouse brain show that J-113397 acts as an antagonist at the ORL_1 receptor when compared to other molecules proposed to be ORL_1 antagonists (Ichikawa et al., 2001). Both studies indicate that J-113397 blocks the ORL_1 receptor in a competitive manner and possesses high affinity and selectivity for the ORL_1 receptor over the other opioid receptors. The findings that nociceptin does not possess abuse liability as it failed to produce place preference or aversion (Devine et al., 1996) could lead to the hypothesis that development of an antagonist that is active in this system and might have therapeutic benefit, without the addictive effects of opiates.

To provide evidence of the selectivity of J-113397, the second part of these experiments involved the i.t. administration of 1 μg of morphine after the administration of 100 μg of J-113397 and here I obtained the marked inhibitory effect

expected by morphine. This demonstrates that J-113397 has no interaction with the mu-opioid receptor.

At this point was necessary to investigate the role of the J-113397 alone and therefore the role of the endogenous nociceptin.

5.3.2 Animals treated with J-113397 alone

The idea that endogenous nociceptin has some basal modulatory role in nociceptive transmission was suggested after reported evidence that nociceptin immuno-reactive like substances can be released from the rat dorsal horn spinal cord (Williams et al., 1998) and overall, the presence of both the peptide and receptor in the brain and spinal cord, for review see (Calo et al., 2000; Mogil and Pasternak, 2001). However to elucidate the role of endogenous nociceptin a selective ORL_1 receptor antagonist was required.

The discovery of the selective nociceptin antagonist J11339 by Kawamoto et al., (1999) and Ozaki et al., (2000) made possible the further investigation of the nociceptin system. In the previous study the interaction between exogenous nociceptin and J-113397 was investigated and maximal inhibition evoked by 250 μg nociceptin was found to be reversed by 100 μg J-113397.

It was now possible for the present electrophysiological study investigating the effects of J-113397 on endogenous nociceptin. In particular I investigated the potential role of endogenously released nociceptin in a rat model of inflammation and neuropathy using J-113397, an antagonist of the ORL_1 receptor. Overall, J-113397 had no significant effect on the electrically or naturally evoked recordings made from deep dorsal horn neurones in normal animals and this did not change after inflammation and neuropathy. Overall, this would indicate that endogenous nociceptin has no or only a minor role in spinal nociceptive processing and this is not altered in chronic pain states.

If endogenous nociceptin had a major role in inhibiting spinal nociception, then this would be blocked by J-113397 thus producing facilitation of either ongoing or evoked dorsal horn neuronal responses but in this study no significant effect was observed. These findings correlate well with studies using a different method of studying endogenous nociceptin; studies where mice strains are produced with specific disruption of genes encoding the ORL_1 receptor or nociceptin. No major change in basal nociception was detected in these mice (Nishi et al., 1997; Mamiya et al., 1998).

5.3.2.1 Endogenous nociceptin in inflammatory pain

The carrageenan model of inflammation was induced in this study to produce a state of persistent inflammatory pain. An injection of carrageenan into the plantar surface of the hindpaw results in localized inflammation and hyperalgesia. In the present study cumulative doses of J-113397 (1, 10 and 100 μg) did not cause any significant change in electrical and mechanical evoked responses in carrageenan treated and normal animals when compared to their respective controls. This could indicate that there is no clear role of the endogenous nociceptin system in normal animals and that it is not modified during acute inflammation. However in carrageenan animals, the increased sensitivity to mechanical (von Frey) stimulus was evident from the baseline response and this was not changed in the presence of J-113397. This enhanced excitability of the neurone in the inflammatory state may reflect changes in the mediators of inflammation such as prostanoids, peptides, ion and receptor operated channels that could increase the observed neuronal responses through peripheral and central sensitization (for further discussion see section 1.6.). However, the enhanced responses seen after inflammation verify that the expected neuronal changes, enhanced responses after carrageenan (itself accompanied by behavioural changes such as allodynia and hyperalgesia) are produced with a time course that parallels the behaviour (Ren and Dubner, 1999).

Previous electrophysiological studies by (Carpenter et al., 2000) investigated the effects of exogenous i.t. nociceptin in the presence of carrageenan induced inflammation. They observed increased inhibitory effects of the peptide agonist on C-fibre evoked and input responses in inflammation compared with normal animals and suggested that increased nociceptin receptor binding in the superficial laminae of the spinal cord after persistent inflammation was a possible explanation (Jia et al., 1998). The results from these reports suggest that ORL_1 receptors are up-regulated following persistent inflammation therefore enhancing the inhibitory actions of nociceptin. The increased levels of nociceptin due to exogenous administration would presumably bind to these ORL_1 receptors that are increased in number producing increased inhibition. It could be suggested that the endogenous nociceptin levels investigated in the present study are insufficient to produce a similar increase in inhibition.

However carrageenan inflammation develops relatively rapidly and the increase in receptor number described in Jia et al., (1998) study was seen 4 days after inflammation as induced by complete Freund's adjuvant (CFA). The effects observed in my study and in Carpenter et al., (2000) were after 3 h. Evidence that the prepronociceptin expression can alter rapidly (within 30min) after carrageenan inflammation has been reported by Andoh et al., (1997) who described increased levels of mRNA message for the nociceptin peptide precursor in the dorsal root ganglion. However no evidence for altered physiological consequences of potential increased endogenous nociceptin was found in the present study.

In my study the lack of any significant change in electrically and naturally evoked responses after J-113397 injection in normal and inflamed animals shows that there is no significant change in the endogenous nociceptin system in those pain states. A lack of specific ORL_1 receptor until now has hampered studies investigating the effects of endogenous nociceptin and so results in this present study cannot be compared to many others. However a recent behavioural study investigating endogenous nociceptin has been reported, using i.t. J-113397 in the rat formalin test, a model of acute inflammatory pain, J-113397 enhanced the formalin-induced

agitation behaviour suggesting it was antagonizing the analgesic effect of endogenously released nociceptin thus producing an algesic effect (Yamamoto et al., 2001). In this present study J-113397 did not produce facilitation of electrically evoked responses indicating an unaltered inhibition of the analgesic effects produced by endogenous nociceptin in carrageenan inflammation.

5.3.2.2 Endogenous nociceptin in neuropathic pain.

As in the previous chapter we have seen that nociceptin has an inhibitory effect on animals with nerve ligation and therefore it could be assumed that ORL_1 receptor is in some way involved in the control of pain transmission following nerve injury. However in the present study I found that both electrical and mechanical induced responses were not modified by the application of cumulative doses of J-113397. Although the highest dose of J-113397 induced a tendency to facilitatory effects on the wind-up and post-discharge, and on the other hand, A-delta, C-fibre and input responses were mildly inhibited by J-113397. The lack of significance in these experiments underlines the absence of an understandable role of the endogenous nociceptin in measures of neuropathic pain. Overall, these experiments suggest that J-113397 has no effect on spinal neuronal responses in neuropathic animals.
Furthermore, in the neuropathic animals, as also seen in sham operated and the carrageenan model of inflammation, I could demonstrate a tendency for increased sensitivity to von Frey stimuli evident from the control situation. This baseline response was higher in the neuropathic and sham rats compared to the normal rats indicating enhancement of the neuronal excitability. The presence of a nerve ligation would presumably result in a markedly reduced input from peripheral stimulation but compensatory mechanisms including transduction changes and structural reorganization have been shown to exist after nerve injury (Chapman et al., 1998).

The result of these experiments cannot be compared to others, since there are very few studies of endogenous nociceptin and none after nerve injury. Therefore I will

discuss studies on the effects of exogenous nociceptin (as also discussed in chapter 3) as a reference. Briefly, studies with exogenous nociceptin have shown the effectiveness of the peptide in neuropathic animals (Yamamoto and Nozaki-Taguchi, 1997; Yamamoto et al., 1997; Hao et al., 1998). Furthermore, in Xu et al., (1999) study the inhibitory effect of nociceptin was found to be increased after nerve injury although this study measured only motor-related function rather than sensory responses. Although the mechanism of nociceptin in neuropathic animals is unknown, Briscini et al., (2002) suggested an up-regulation of ORL_1 receptors in the spinal cord or dorsal root ganglia in these animals. However, this mechanism is not reflected in this study, possibly because the endogenous nociceptin levels were not enough to bind to these increased receptors and produce increased inhibition, but also as I saw that high concentrations of exogenous nociceptin were no more effective in this pain state when compared with normal animals (further discussed in chapter 3).

An important point to consider is the differences between nociceptin and the other opioid systems. It is well known that peptides such as CCK are upregulated in neuropathic pain and they are thought to be one of the main causes of the altered opioid actions due to their anti-opioid effect in those pain states. The next chapter will investigate the effect of spinal CCK on nociceptin systems.

In conclusion, the findings of these experiments indicate a minimal role for endogenous nociceptin in the spinal processing of pain and this action is unaltered in the presence of inflammation or neuropathy. Although the former was studied over 3-6 hours, the neuropathy studies were done at 2 weeks which would be long enough for new protein to be produced (precursor or receptor).

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CHAPTER 6

INTERACTIONS BETWEEN NOCICEPTIN AND CHOLECYSTOKININ

6.1 INTRODUCTION

The predominant form of cholecystokinin (CCK) found in the mammalian central nervous system is the carboxy-terminal octapeptide (CCK-8). CCK-8 is the ligand for the CCK receptor, there are two types of receptor, CCK_A or CCK_1 (peripheral type) and CCK_B or CCK_2 mainly present in the central nervous system (Moran et al., 1986), especially in the rat (Hill and Woodruff, 1990).

The wide distribution of CCK in the central nervous system (Williams et al., 1987; Schiffmann and Vanderhaeghen, 1991), in particular, in the superficial laminae of the dorsal horn of the spinal cord, suggests an important role of this peptide in the modulation of nociceptive transmission. In the first studies defining the physiological role for CCK as a functional antagonist of opioid-induced analgesia (i.e. 'anti-opioid') it was demonstrated that CCK significantly reduces morphine analgesia in the rat tail flick test (Faris et al., 1983). In addition, CCK receptor antagonists induce enhancement of opioid analgesia (Watkins et al., 1985; Stanfa et al., 1994).

In most experiments CCK does not alter baseline pain thresholds, so it is clear that the blockade of morphine analgesia is not due to a direct hyperalgesic effect of CCK (Xu and Wiesenfeld-Hallin, 1997). Although the mechanism is not fully understood by which CCK antagonises opioid analgesia, there is good evidence that CCK counteracts intracellular events subsequent to opioid activation through calcium levels (Wang et al., 1992).

CCK may play a role in the physiological determination of opioid actions since in a rat model of neuropathic pain, there is an increase in spinal CCK and a reduction in the potency of spinal morphine (Xu et al., 1993). This may partly explain cases of reduced opioid sensitivity of neuropathic pain in man. In this chapter I will investigate whether CCK, known to modulate the other mu, delta and kappa opioid systems will also have effect on the actions of nociceptin. This interaction between

CCK and the ORL_1 receptor has never been tested at spinal levels. I have therefore studied the CCK-ORL_1 interaction after nerve injury, in sham operated and in normal animals.

6.2. RESULTS

The interactions between CCK and nociceptin were studied in normal (n=6), sham-operated (n=6) and neuropathic rats (n=6). In addition I also used a group of normal (n=6), sham (n=6) and neuropathic rats (n=6) treated only with nociceptin (data from chapter 3).

CCK 1 μg was given intrathecally 20 min before the application of 250 μg of nociceptin and the neuronal responses were followed until maximum inhibition was achieved (normally 1 h). The group treated with only nociceptin 250 μg was also followed until maximum effects were achieved. A total of 18 dorsal horn neurones were studied in this experiment and their mean depth was 750 ± 50 μm from the surface of the spinal cord and this did not differ between groups.

In this study was employed Mann-Whitney test for the comparison of drug effects between animal groups and the level of significance was taken to be $P \leq 0.05$.

6.2.1 Nociceptin alone

In all three animal groups (normal, sham operated and neuropathic) intrathecal injection of nociceptin 250 μg did not cause changes in Aβ induced activity (data not shown). In normal and neuropathic rats nociceptin caused a clear inhibition of C-fibre evoked responses (normal 70.8 ± 9%, neuropathic 39.3 ± 4%), post-discharge (normal 89 ± 6%, neuropathic 65.7 ± 10%), wind-up (normal 72.9 ± 9%, neuropathic 69 ± 11%) and input (normal 87 ± 10%, neuropathic 41 ± 22%) see Fig.1–4 (A). Although there was a tendency for the effects of nociceptin to be reduced after nerve injury this was not significant when compared to normal animals (using Mann-Whitney test). Contrarily in sham operated rats, nociceptin did not have any

inhibitory effect but rather facilitated the wind-up by +64 ± 9% and post-discharges increased by +15±10%, both significant ($P \leq 0.05$) when compared to the normal animals Fig.1–4 (A).

6.2.2 CCK and nociceptin

The application of 1 μg of CCK did not cause a significant effect in any of the three groups although there was a tendency towards a mild facilitation (data not shown). After the application of 250 μg of nociceptin in the continued presence of CCK it was observed that in normal animals, there were still no effects of nociceptin on Aβ- and Aδ-fibre evoked responses. However, in normal animals the marked inhibitory effect of nociceptin alone on C-fibre, post-discharge, wind-up and input responses was abolished in the presence of CCK (see Fig.1–4 respectively). Thus, CCK acts as an anti-opioid at the ORL_1 receptor in normal rats as shown by its block of the effect of nociceptin.

In sham operated rats where the application of nociceptin alone did not cause any inhibition, nociceptin in the presence of CCK caused a significant ($P \leq 0.01$) inhibition of the C-fibre evoked responses from +1.9 ± 7.6% facilitation in normal to 62 ± 14% inhibition in sham animals with CCK/nociceptin, see Fig.1B. Also wind-up was significantly ($P \leq 0.01$) inhibited to a much greater extent comparing a +27 ± 35% facilitation in normal animals to 80 ± 7% inhibition in sham rats with CCK plus nociceptin, see Fig.3B. There was also significant ($P \leq 0.05$) inhibition of input responses in sham rats after the combination with respect to lack of effect the normal animals, see Fig.4 (B).

The inhibitory effect of nociceptin after CCK on the C-fibre, post discharge, wind-up and input, response was considerably greater in neuropathic rats and caused an almost complete block of activity (86 ± 4%, 97 ± 2%, 88 ± 6% and 96 ± 3% respectively) leading to a significant inhibition ($P \leq 0.01$ in all cases), see Fig.1-4 (B).

Comparison of the effect of 250 μg of nociceptin in sham animals and the neuropathic group, both pre-treated with 1 μg of CCK, reveals no difference between the groups although they have opposite responses when treated with only nociceptin.

Overall, in the presence of CCK, the inhibitory effect of nociceptin on C-fibre, post-discharge, wind-up and input, is blocked in normal animals. On the other hand, in the presence of CCK the effect of nociceptin is not only restored in sham rats but is enhanced in the neuropathic group.

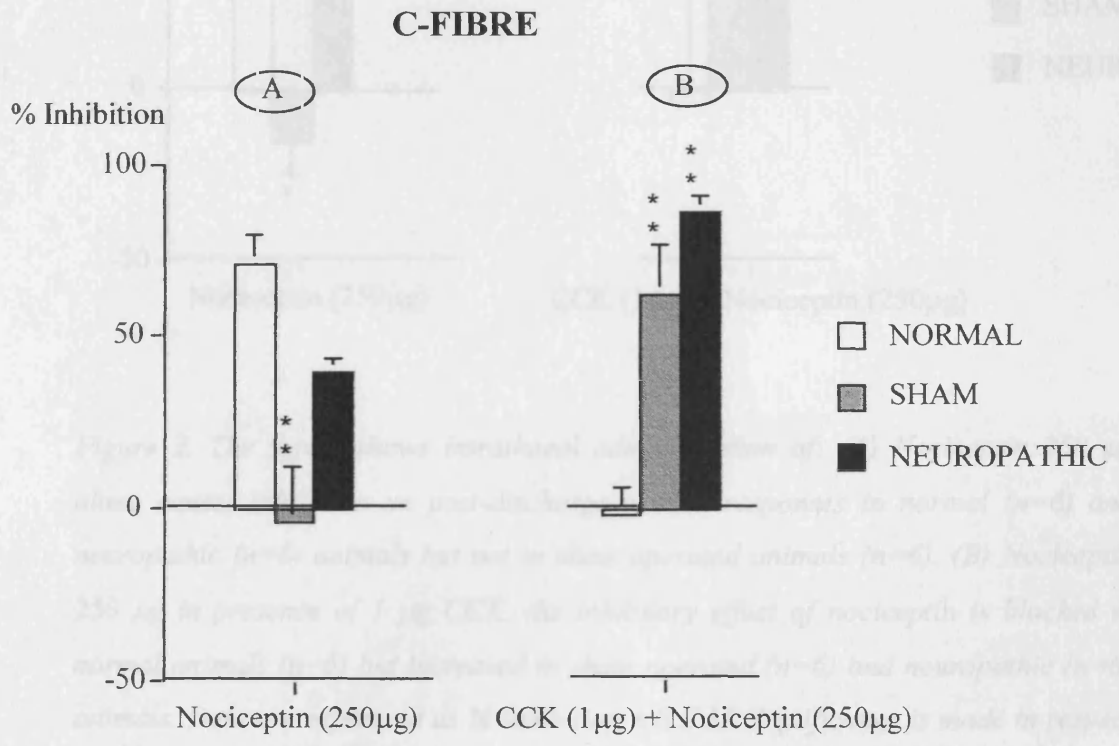

*Figure 1. Intrathecal administration of: (A) Nociceptin 250 μg alone causes inhibition on C-fibres evoked responses in normal (n=6) and neuropathic (n=6) animals but not in sham operated animals (n=6). (B) Nociceptin 250 μg in presence of 1 μg CCK. The inhibitory effect of nociceptin is blocked in normal animals (n=6) but increased in sham operated (n=6) and neuropathic (n=6) animal by CCK. Data are expressed as % inhibition ± S.E.M. Significance is made in respect of the normal group (**P≤0.01 using Mann-Whitney test).*

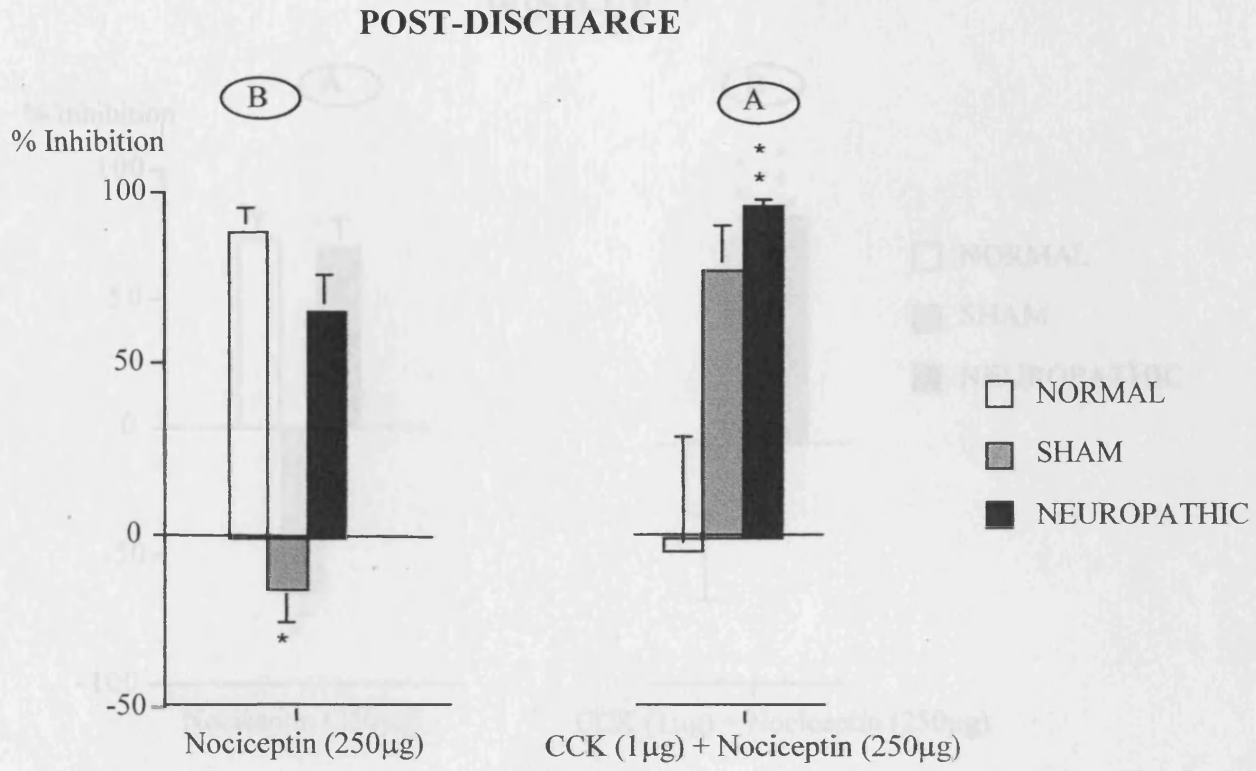

*Figure 2. The figure shows intrathecal administration of: (A) Nociceptin 250 µg alone causes inhibition on post-discharge evoked responses in normal (n=6) and neuropathic (n=6) animals but not in sham operated animals (n=6). (B) Nociceptin 250 µg in presence of 1 µg CCK, the inhibitory effect of nociceptin is blocked in normal animals (n=6) but increased in sham operated (n=6) and neuropathic (n=6) animals. Data are expressed as % inhibition ± S.E.M. Significance is made in respect of the normal group (**P≤0.01, *P≤0.05 using Mann-Whitney test).*

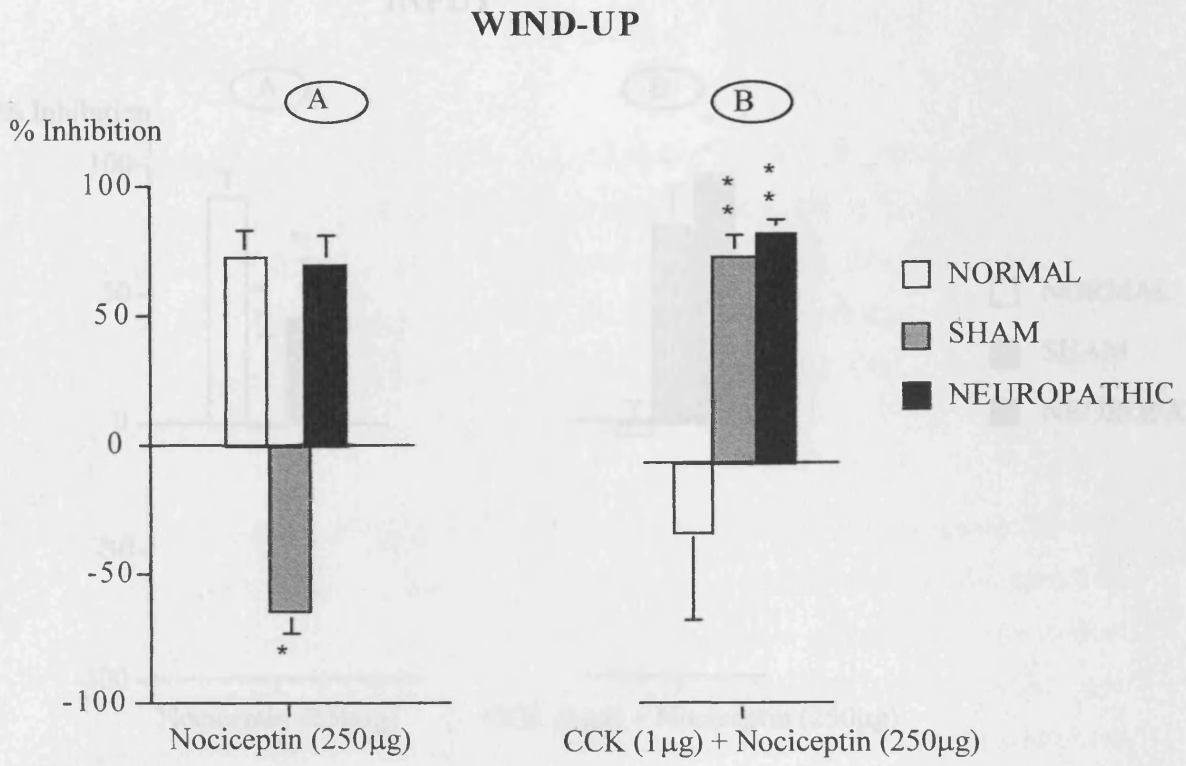

*Figure 3. Intrathecal administration of: (A) Nociceptin 250 μg alone causes inhibition on wind-up evoked responses in normal (n=6) and neuropathic (n=6) animals, while induces facilitation in sham operated animals (n=6). (B) Nociceptin 250 μg in presence of 1 μg CCK, the inhibitory effect of nociceptin is blocked in normal animals (n=6) but increased in sham operated (n=6) and neuropathic (n=6) animals. Data are expressed as % inhibition ± S.E.M. Significance is made in respect of the normal group (**P≤0.01, *P≤0.05 using Mann-Whitney test).*

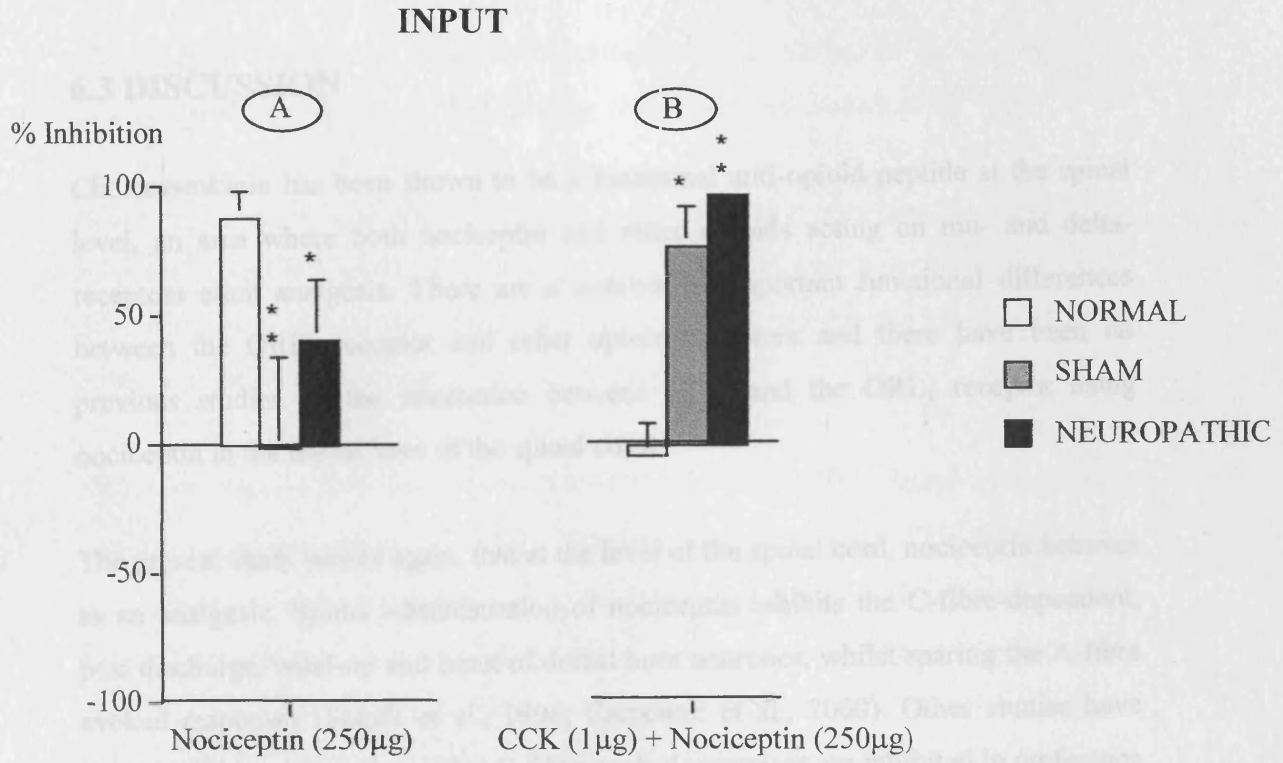

*Figure 4. Intrathecal administration of: (A) Nociceptin 250 µg alone causes inhibition on input evoked responses in normal (n=6) and neuropathic (n=6) animals but not in sham operated animals (n=6). (B) Nociceptin 250 µg in presence of CCK 1 µg, the inhibitory effect of nociceptin is blocked in normal animals (n=6) but increased in sham operated (n=6) and neuropathic (n=6) animals. Data are expressed as % inhibition ± S.E.M. Significance is made in respect of the normal group (**P≤0.01, *P≤0.05 using Mann-Whitney test).*

6.3 DISCUSSION

Cholecystokinin has been shown to be a functional anti-opioid peptide at the spinal level, an area where both nociceptin and other opioids acting on mu- and delta-receptors elicit analgesia. There are a number of important functional differences between the ORL_1 receptor and other opioid receptors and there have been no previous studies on the interaction between CCK and the ORL_1 receptor using nociceptin in the dorsal horn of the spinal cord.

The present study shows again, that at the level of the spinal cord, nociceptin behaves as an analgesic. Spinal administration of nociceptin inhibits the C-fibre-dependent, post discharge, wind-up and input of dorsal horn neurones, whilst sparing the A-fibre evoked responses (Stanfa et al., 1996; Carpenter et al., 2000). Other studies have supported this with evidence that C-fibre evoked responses are inhibited in preference to A-fibre evoked responses (Faber et al., 1996), so that the peptide has selective effects on noxious evoked activity and hyperalgesia (Hao et al., 1998). This suggests a selective action of nociceptin on noxious evoked responses in agreement with previous studies (Faber et al., 1996; Candeletti et al., 2000; Carpenter et al., 2000) and my studies in this thesis. Here it was also found that nociceptin had the expected spinal analgesic effect in normal and neuropathic rats, although I did not observe any analgesic effect in sham operated rats. Clearly there is plasticity in the effects of nociceptin and although the sham surgery is an essential control for the neuropathic model, we have observed changes in the effect of another peptide, galanin, in this condition (Flatters et al., 2002). There are clearly persistent changes that may represent post-operative pain conditions, after the sham surgery since it involves anaesthesia, exposure of nerve, suturing and recovery is possibly not surprising.

In this study, I found that application of CCK did not cause a significant effect on neuronal activity in any of the three groups in agreement with the results of Stanfa and Dickenson, (1993) in normal animals and after carrageenan inflammation. In normal animals CCK had an expected 'anti-opioid' action since it blocked the effects

of nociceptin in a similar manner to that reported for mu-opioid receptor agonists such as morphine (Faris et al., 1983; Baber et al., 1989; Magnuson et al., 1990).

However, I found that CCK enhanced the inhibitory effects of nociceptin in neuropathic and in sham rats, comparing to rats treated with only nociceptin. Thus CCK does not act as an anti-opioid after either sham surgery or the subsequent nerve injury but rather enhances the action of nociceptin.

Thus there are similarities between spinal mu-opioid and ORL_1 receptor function in normal animals and after tissue injury. However, after nerve injury CCK had no 'anti-opioid' action since it failed to block the effects of nociceptin and in fact, tended to augment the inhibitory effects of the peptide. This is entirely different from morphine since in a rat model of neuropathic pain, there is an increase in spinal CCK and a reduction in the potency of spinal morphine (Xu et al., 1993). Other studies have also shown reduced effects of systemic but not spinal morphine after nerve injury in this electrophysiological model (Suzuki et al., 1999). Since this profile of the CCK-nociceptin interaction is very different from that of morphine (Stanfa and Dickenson, 1993), the results suggests a plasticity in the actions of CCK/nociceptin and further emphasises that the ORL_1 receptor has very different characteristics from the mu-opioid receptor at spinal levels. It is interesting to note that these marked changes in the inhibitory effect of nociceptin in the presence of CCK in neuropathic and sham rats occurs in situations were there is an upregulation of both the CCK receptor and ORL_1 receptor (Xu et al., 1993; Briscini et al., 2002) respectively.

Work from this laboratory has also shown that gabapentin, an anti-convulsant used for neuropathic pain patients also has enhanced effects after both sham and nerve injury as compared to normal animals (Chapman et al., 1998).
One of the most widely proposed mechanisms of CCK is an involvement in calcium signalling. Intracellular calcium is mobilised by CCK, which opposes the suppression mediated by opioids of the increase in intracellular calcium. Furthermore, CCK reverses the suppression of the K^+-induced increase in intracellular calcium produced

by opioids (Wang et al., 1992). Since gabapentin is thought to modulate calcium channel activity and there is an upregulation of the alpha 2 delta subunit of calcium channels after nerve injury and enhanced actions of both N (nerve injury) and P (tissue damage) after pathological changes (Dickenson et al., 2002), this suggests that the CCK/ORL_1 interaction, as suggested for mu-opioid receptors in normal animals may involve intracellular calcium. Another possible mechanism is that CCK, acting on CCK_B receptors, decreases the availability of the enkephalins, possibly by inhibiting the release or increasing the metabolism of the enkephalins (Nichols et al., 1996).

Whatever the form of the interaction, in normal animals CCK plays a physiological role in negatively modulating antinociceptive action of opioid agonists in the spinal cord. Since this role is altered after nerve injury and in presence of surgery, the ORL_1 receptor may be a target for the treatment of nerve injury and other pain states but this will require production of a non-peptide agonist at this receptor.

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CHAPTER 7

THE SPINAL EFFECTS OF NOCICEPTIN AND DPDPE IN SAP AND SP-SAP ANIMALS

7.1 INTRODUCTION

One of the most important goals in the study of pain is in the understanding of sensory processing in the spinal cord and subsequently to be able to recognize circuits underlying the transmission of information from nociceptive afferents to the brain. Progress so far has been limited due to the complex neuronal circuitry within the dorsal horn. A key issue would be to understand the function of cells in lamina I of the spinal cord which are thought to have a particular important role in nociception since this region is one of the main recipient areas for Aδ- and C-fibre afferent inputs (Sorkin and Carlton, 1997). Lamina I neurones are also known to be the main projection neurones from the superficial layer, which projects to the brainstem areas that are important in the central processing of nociceptive information (Craig and Dostrovsky, 1997; Todd et al., 2002).

A sub-population of neurones of lamina I expressing the NK1 receptor are believed to play an important role in this process and in recent years several studies have been directed towards elucidating the roles of these neurones in nociceptive signaling. Although these neurones constitute less than 10% of all lamina I neurones (Brown et al., 1995; Mantyh et al., 1997), many of them project to multiple sites within the brainstem (Mantyh et al., 1997; Todd et al., 2000). In particular, the main supraspinal target of this projection is the parabrachial (PB) area, in fact the spinoparabrachial pathway constitutes a major link between the spinal cord and the brainstem, and has neurones implicated in the affective aspect of pain processing (Bernard et al., 1996). Also the spinothalamic pathway presents an important pathway involved in sensory discrimination aspects of pain (Gauriau and Bernard, 2002), yet in this pathway only few lamina I neurones project directly to the thalamus.

Moreover these neurones are activated by substance P (SP) previously described in chapter 1. Briefly, SP, an 11-amino-acid peptide contained in small diameter primary afferent fibres is released into the dorsal horn upon noxious stimulation (Duggan et al., 1988). Substance P by acting on the NK1 receptor activates nociceptive-specific

dorsal horn neurones and so induces an excitatory effect possibly by synergism with the excitatory amino acids (Xu et al., 1992). Moreover, most spinal NK1 expressing neurones in lamina I do not contain GABA or glycine receptor and therefore these neurones are excitatory neurones within the spinal cord (Littlewood et al., 1995).

In recent years an interesting technique has been developed for the investigation of lamina I NK1-expressing neurones in nociceptive signaling. Based on the characteristic of internalization of G-protein-coupled-receptors, following noxious peripheral stimulation the SP released binds to the spinal dorsal horn neurones expressing the NK1 receptor, and subsequently both SP and NK1 receptor are rapidly internalized (Bowden et al., 1994; Mantyh et al., 1995). Mantyh and colleagues (1997) used this to develop a technique to ablate these lamina I neurones. The authors infused into the spinal cord SP conjugated with the ribosome inactivating- protein saporin (SAP), so that the SP-SAP complex was internalized only by the subpopulation of neurones that express the NK1 receptor. As a consequence of the internalization of SAP, the neurones died within 28 days since normal cell function is blocked as a result of ribosomal inactivation. Subsequently, they found that animals treated with SP-SAP where these lamina I neurones are ablated, exhibit a significant attenuation of mechanical and heat hyperalgesia produced by intraplantar injection of capsaicin, while in animals treated with saline, SP or SAP alone there are no significant changes. The data therefore suggest that these neurones play an important role in the behavioral nociceptive responses (Mantyh et al., 1997).

In the light of these findings and by using the same technique I investigated the effects of nociceptin and the delta-opioid ligand, DPDPE, using electrophysiological evoked responses of deep dorsal horn neurones in these lamina I NK1 depleted animals. Taking into account the importance of these lamina I neurones in the control of pain, the present experiment was designed to understand the influence if any of the ablation of the lamina I NK1 expressing neurones on the function of the nociceptin receptor system on spinal processing. Since opioid receptors are found both pre- and post-synaptically in spinal circuits, on afferent terminals and spinal neurones within

the superficial spinal cord, it seemed important to determine if removal of the main neuronal population expressing post-synaptic opioid receptors had an effect on spinal opioid analgesia.

Nociceptin, the ORL_1 agonist as previously described in chapter 3, here again was the central aim of this study. The other compound in the study is DPDPE ([D-Pen^2,D-Pen^5]-enkephalin), a highly selective delta-opioid receptor agonist (Stewart and Hammond, 1993; Kieffer, 1997). The predominant pre-synaptic effect of this compound (Rahman and Dickenson, 1999) made it interesting to investigate whether the effect of DPDPE on deep dorsal horn neurones would be effected by the lack of lamina I neurones expressing NK1 receptors and post-synaptic opioid receptors.

7.2 RESULTS

Thirty eight dorsal horn neurones with mean depth 770 ± 50 μm were studied in this experiment. As already described in the methods section, 28 days before the electrophysiological study all animals were given a single dose of intrathecal saline, SP-SAP or SAP and named as saline group (n=12), SP-SAP group (n=14) and SAP group (n=12) respectively. From day 28, the rats were used for the electrophysiological studies where half of the animals were treated with increasing doses of intrathecal nociceptin (5, 50, 125 and 250 μg) and in a similar way the remaining were treated with 1, 10, 50 and 100 μg of DPDPE.

Electrophysiological studies of the effect of nociceptin (5, 50, 125 and 250 μg) showed no inhibition other than tendency of facilitation for the A-beta response in three animal groups (see Fig.1). In contrast the Aδ- and C-fibre evoked activity show combinations of changes in activity after nociceptin which included both facilitatory and inhibitory effects. Both afferent fibre evoked responses were facilitated or unaltered with lower doses of nociceptin (5 and 50 μg) while higher doses of nociceptin induced pronounced inhibition. For example, nociceptin 5 μg produces no change (2 ± 8%) on C-fibre evoked response in SAP animals while the top dose 250

μg induces an inhibition of 40 ± 13% in the same animals (Fig.2). Furthermore lower doses of nociceptin caused facilitation of post-discharge, wind-up and input responses whereas with the higher dose of nociceptin the inhibition was more prominent in all animal groups (Fig.2.) On these responses, the SAP and saline groups show the major inhibitory effect when compared to NK1 depleted animals, in fact with nociceptin 250 μg the wind-up response reached up to 82 ± 11% of inhibition with $P< 0.05$ and 77 ± 32% when compared to the respective pre-drug control values.

Overall the data shows that nociceptin induces similar effect in saline and SAP animals, suggesting that these two animal groups are similar. However in SP-SAP animals there is a tendency for an attenuation in the effect of spinal nociceptin when compared to saline and SAP animals. This reduced nociceptin effect in SP-SAP animals although was not significant was present on C-fibre and input evoked response as well as the wind-up response where the greatest difference (almost 60%) between the two animal groups was seen.

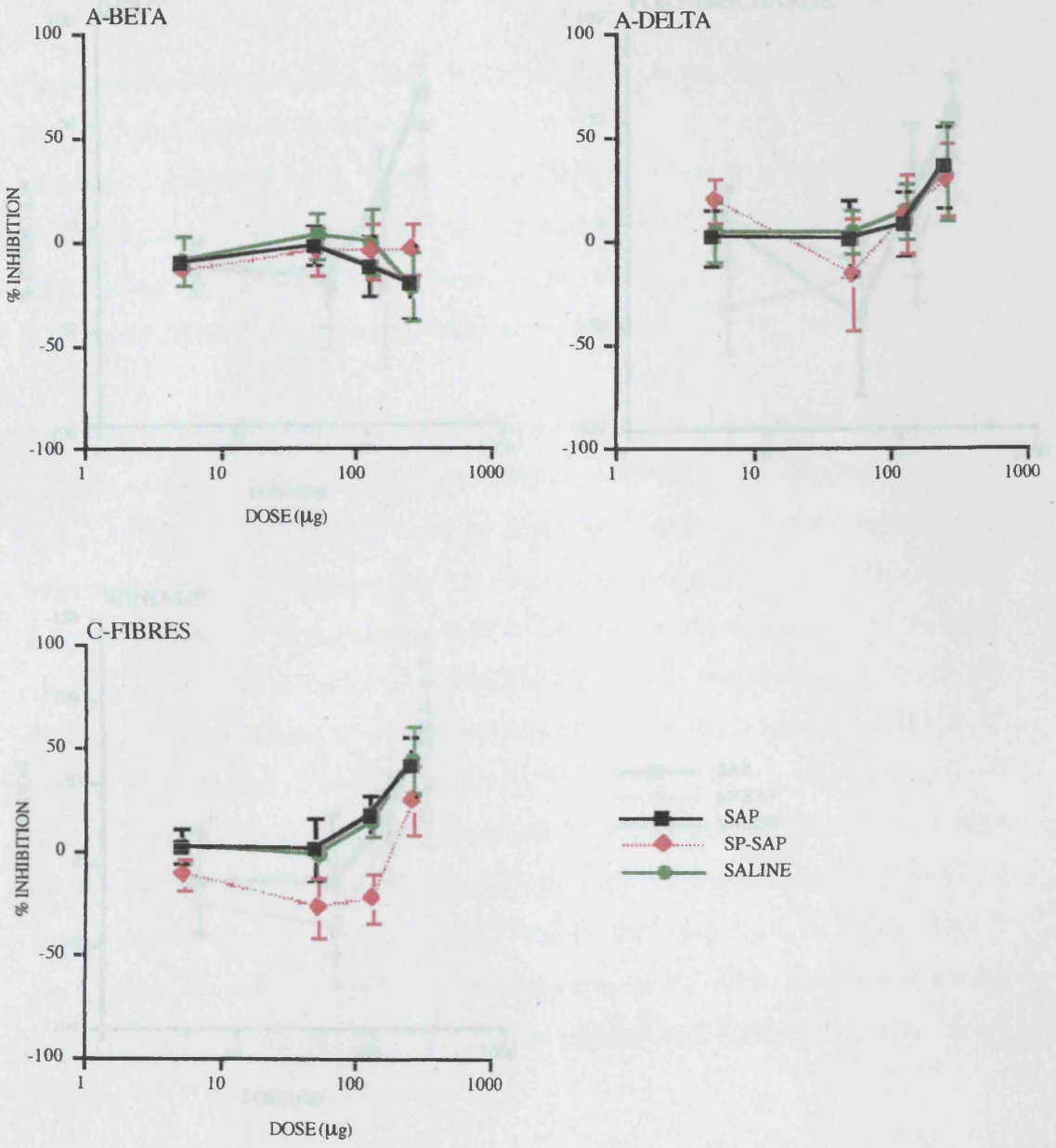

Figure 1. Comparison of the effect of spinally applied nociceptin (5, 50, 125 and 250 µg) on Aβ-, Aδ- and C-fibre evoked responses from saline (n=6), SAP (n=6) and SP-SAP (n=7) animals. Data were expressed as % inhibition ± SEM. No significance was found when compared effects of the individual doses to pre-drug control values as well as when compared saline versus SAP and SP-SAP animals (Tukey and ANOVA tests).

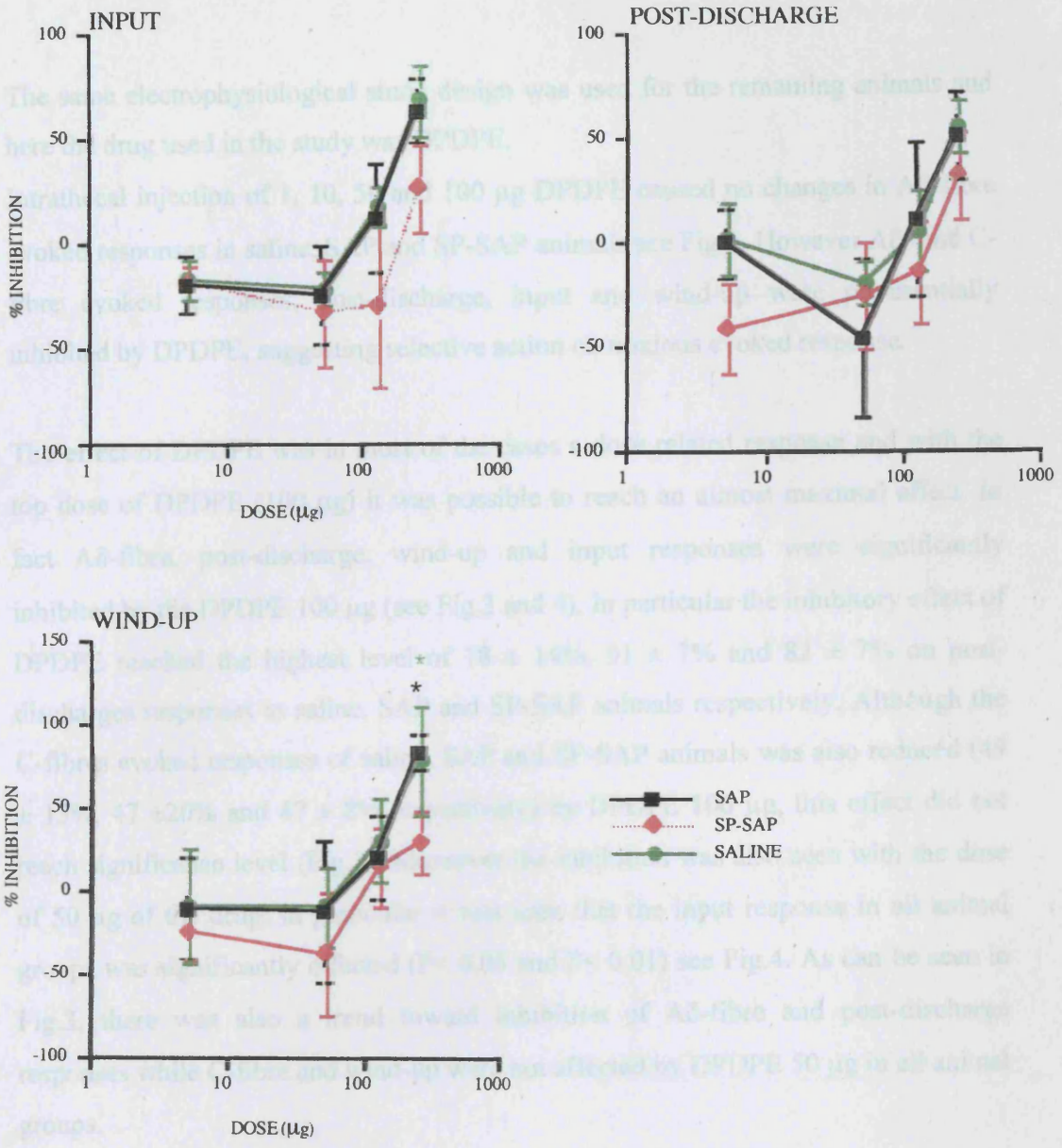

*Figure 2. Comparison of the effect of spinally applied nociceptin (5, 50, 125 and 250 µg) on Input, Post-discharge and Wind-up responses from saline (n=6), SAP (n=6) and SP-SAP (n=7) animals. Data were expressed as % inhibition ± SEM. Significance was found in respect of the corresponding pre-drug control values (*P≤ 0.05 by Tukey and ANOVA tests).*

The same electrophysiological study design was used for the remaining animals and here the drug used in the study was DPDPE.
Intrathecal injection of 1, 10, 50 and 100 μg DPDPE caused no changes in Aβ-fibre evoked responses in saline, SAP and SP-SAP animals see Fig.3. However Aδ- and C-fibre evoked responses, post-discharge, input and wind-up were preferentially inhibited by DPDPE, suggesting selective action on noxious evoked response.

The effect of DPDPE was in most of the cases a dose related response and with the top dose of DPDPE (100 μg) it was possible to reach an almost maximal effect. In fact Aδ-fibre, post-discharge, wind-up and input responses were significantly inhibited by the DPDPE 100 μg (see Fig.3 and 4). In particular the inhibitory effect of DPDPE reached the highest level of 78 ± 14%, 91 ± 7% and 82 ± 7% on post-discharges responses in saline, SAP and SP-SAP animals respectively. Although the C-fibres evoked responses of saline, SAP and SP-SAP animals was also reduced (49 ± 15%, 47 ±20% and 47 ± 8% respectively) by DPDPE 100 μg, this effect did not reach significance level (Fig.3). Moreover the inhibition was also seen with the dose of 50 μg of the drug; in particular it was seen that the input response in all animal groups was significantly effected ($P< 0.05$ and $P< 0.01$) see Fig.4. As can be seen in Fig.3, there was also a trend toward inhibition of Aδ-fibre and post-discharge responses while C-fibre and wind-up were not affected by DPDPE 50 μg in all animal groups.

Following the maximum inhibition obtained after DPDE (100 μg) in the different animal groups, the effects of the drug were reversed by intrathecal injection of naloxone (50 μg) and the neuronal response returned to the control values (data not shown).

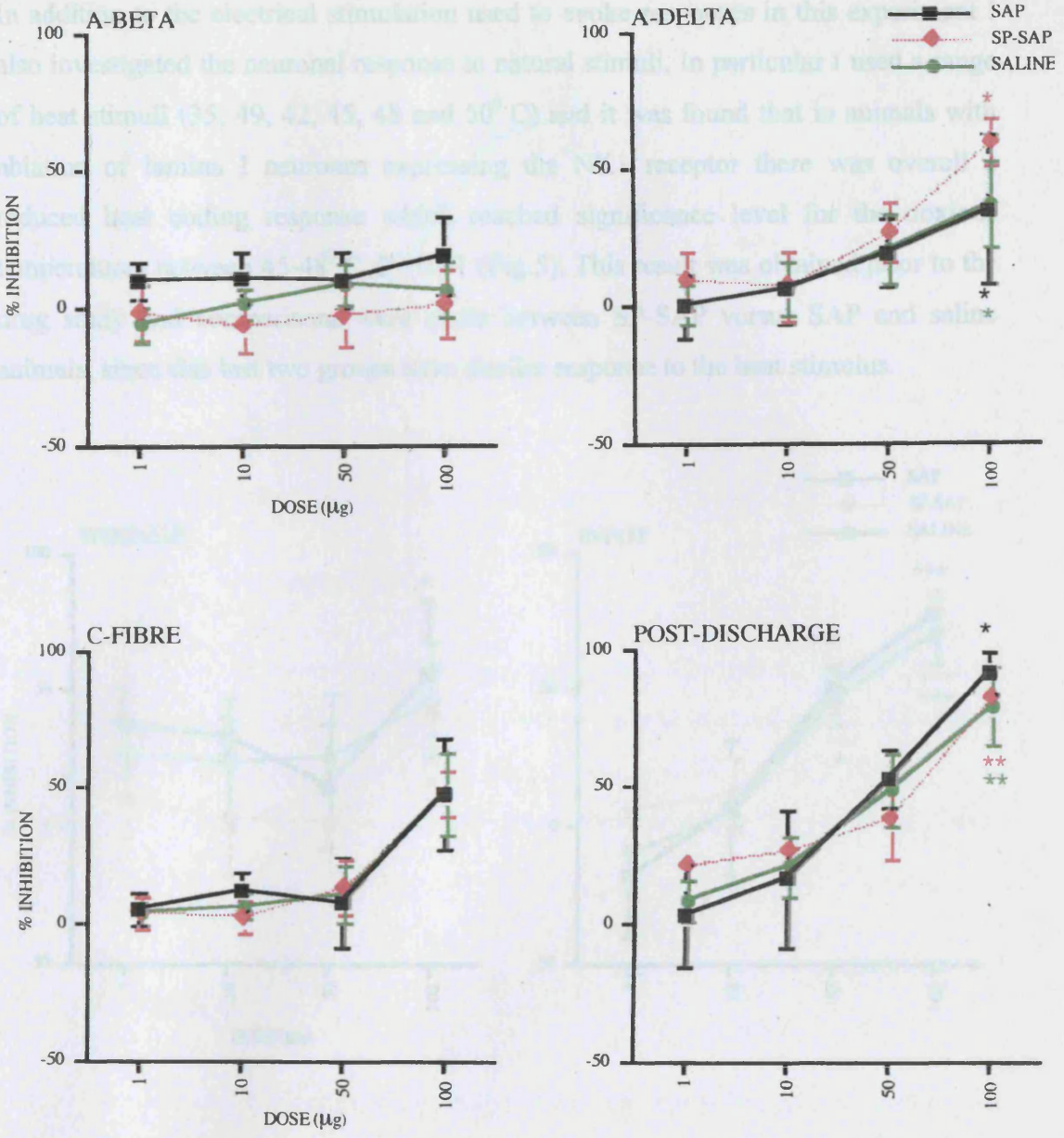

*Figure 3. Effect of spinal administration of DPDPE on electrical evoked responses of dorsal horn neurones from saline (n=6), SAP (n= 6) and SP-SAP (n= 7) animals. Data were expressed as % inhibition ± SEM. Significance was found in respect of the corresponding per-drug control values (**P≤ 0.01, *P≤ 0.05 by Tukey and ANOVA tests).*

In addition to the electrical stimulation used to evoke responses in this experiment I also investigated the neuronal response to natural stimuli. In particular I used a range of heat stimuli (35, 49, 42, 45, 48 and 50^0 C) and it was found that in animals with ablation of lamina I neurones expressing the NK1 receptor there was overall a reduced heat coding response which reached significance level for the noxious temperatures between 45-48^0 C, $P< 0.01$ (Fig.5). This result was obtained prior to the drug study and comparisons were made between SP-SAP versus SAP and saline animals, since this last two groups have similar response to the heat stimulus.

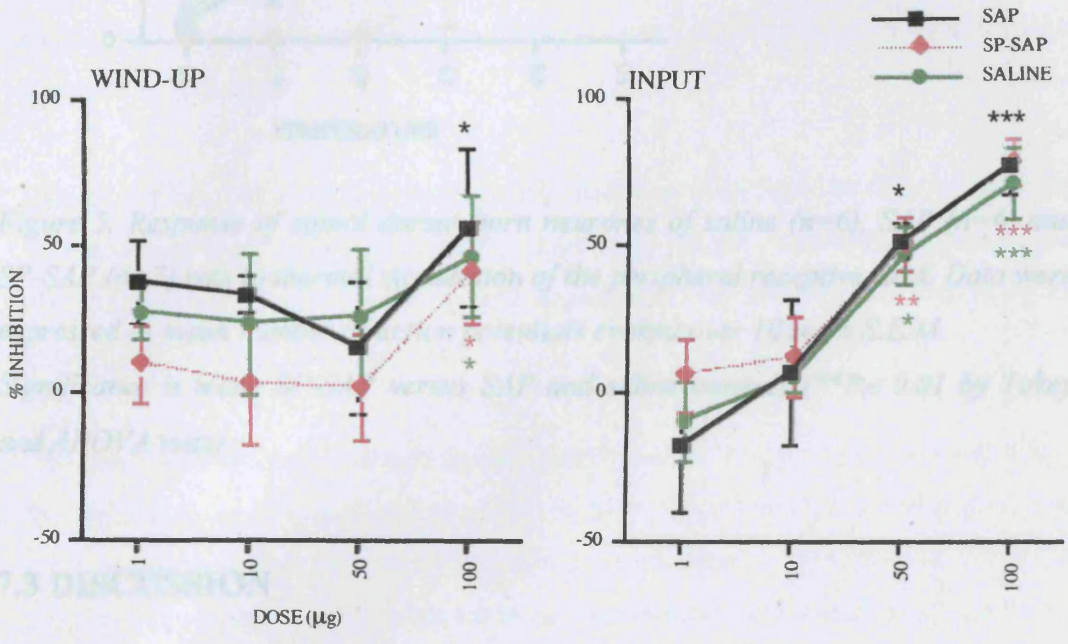

*Figure 4. Comparison of the effect of spinally applied DPDPE on Wind-up and Input evoked responses from saline (n=6), SAP (n=6) and SP-SAP (n=7) animals. Data were expressed as % inhibition ± SEM. Significance was found versus the corresponding pre-drug control values (***P≤ 0.001, **P≤ 0.1, *P≤ 0.05 by Tukey and ANOVA tests).*

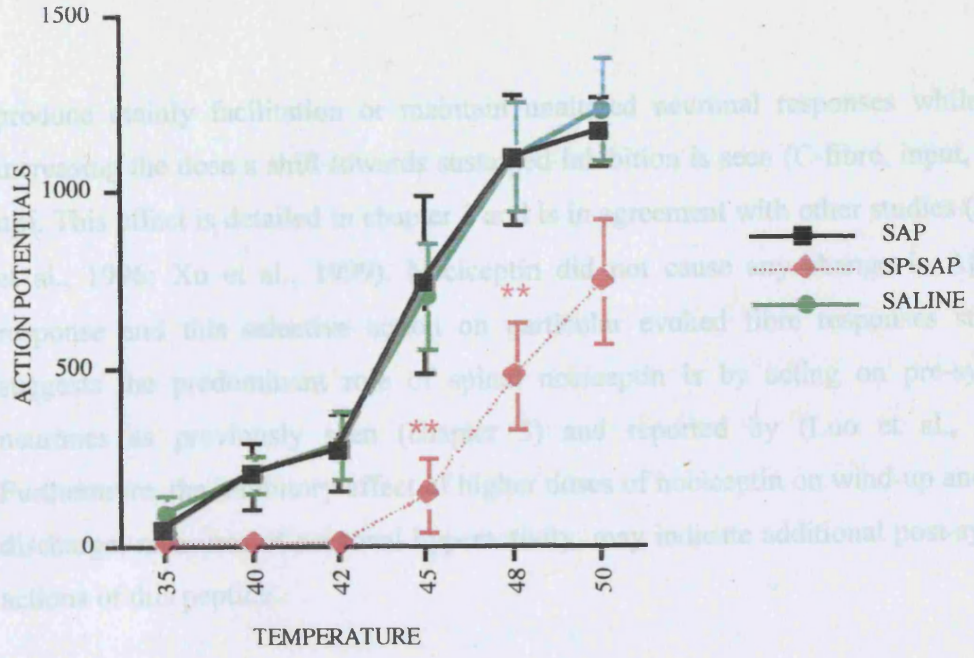

*Figure 5. Response of spinal dorsal horn neurones of saline (n=6), SAP (n=6) and SP-SAP (n=7) rats to thermal stimulation of the peripheral receptive field. Data were expressed as mean number of action potentials evoked over 10 sec ± S.E.M. Significance is made SP-SAP versus SAP and saline animals (**P≤ 0.01 by Tukey and ANOVA tests).*

7.3 DISCUSSION

This is the first electrophysiological study addressing the effect of nociceptin and DPDPE in the spinal processing of sensory information in animals with ablated lamina I neurones expressing the NK1 receptor and using saline or SAP animals as a control group, since the two groups have similar evoked responses. This technique uses site-specific ablation of pharmacologically defined neuronal populations, and so is a powerful approach to examining the roles of particular neurones in function within integrated systems.

Intrathecal nociceptin depending on the dose used, exerts effects that lead to facilitation and inhibition of deep dorsal horn neurones. Low doses of nociceptin

produce mainly facilitation or maintain unaltered neuronal responses while with increasing the dose a shift towards sustained inhibition is seen (C-fibre, input, wind-up). This effect is detailed in chapter 3 and is in agreement with other studies (Stanfa et al., 1996; Xu et al., 1999). Nociceptin did not cause any change in Aβ-fibre response and this selective action on particular evoked fibre responses strongly suggests the predominant role of spinal nociceptin is by acting on pre-synaptic neurones as previously seen (chapter 3) and reported by (Luo et al., 2002). Furthermore, the inhibitory effect of higher doses of nociceptin on wind-up and post-discharge, measures of neuronal hyperactivity, may indicate additional post-synaptic actions of this peptide.

One of the key points of this study was to elucidate both how the deep dorsal horn neuronal responses were altered by the absence of lamina I neurones expressing the NK1 receptor and then how this would impact upon the effects of activation of the ORL-1 and delta-opioid receptors.

The present study shows that in animals with ablated lamina I neurones expressing the NK1 receptor, the neuronal responses to heat were attenuated which agrees well with previous studies (Suzuki et al., 2002). In addition, I show that the effect of intrathecal nociceptin on deep dorsal horn neurones was attenuated when compared with the control group (saline and SAP). The difference was at the highest level (about 60%) for the wind-up response in the SP-SAP group after spinal application of nociceptin at top dose (250 μg).

Although it is well known that the lamina I neurones expressing NK1 receptor represent only a small proportion of the lamina I neuronal population (Brown et al., 1995; Marshall et al., 1996; Mantyh et al., 1997) they are important in the ascending conduction of nociceptive information (Mantyh et al., 1997) due to their predominant projection to a number of supraspinal sites. However, it was found in this study no marked change on the effect of nociceptin on deep dorsal horn neurones. The response that is mostly influenced was the wind-up response, which is an intrinsic

spinal phenomenon relying on lamina I-lamina V circuitry (Dickenson et al., 2004) and so would be most subject to modulation by the missing opioid receptors on post-synaptic neurones.

To further analyze the importance of the lamina I neurones on the response of deep dorsal horn neurones to opioids the second part of these experiments involves the use of DPDPE. This was chosen not simply as a comparator acting on another opioid receptor system but because it is also peptide in nature and so might have a similar pharmacokinetics to nociceptin.

DPDPE induced a dose related inhibition of the electrical evoked response in saline, SAP and SP-SAP animals. The highest dose of 100 μg DPDPE induced the greatest inhibition on post-discharge evoke response in those animal groups while Aβ-fibre respones was the least inhibited. This spared inhibition of DPDPE on Aβ-fibre responses compared to Aδ-and C-fibre, suggest a predominant effect of DPDPE on pre-synaptic sites, previously reported by Rahman and Dickenson, (1999). Moreover, both in Rahman's and the present study it was shown that DPDPE has an additional post-synaptic effect as it was able to inhibit post-synaptic mechanisms such as post-discharge and wind-up evoked responses. The action of DPDPE on post-synaptic sites could be explained by the ability of opioids to extend their inhibitory actions to post-synaptic neurones when administrated at higher doses (Yaksh and Noueihed, 1985) and also by the high distribution (75%) of opioids in the pre-synaptic neurones on the central terminals of the primary afferent fibres leaving 25% of receptors on spinal neurones (Dickenson, 2001).

The inhibitory effect of DPDPE was mediated by an opioid receptor since this inhibition was reversed by naloxone. In particular several studies have shown that the spinal effect of DPDPE is through delta-opioid receptor (Dickenson et al., 1987; Stewart and Hammond, 1993; Kieffer, 1997). Similar to the other opioids the analgesic effect delta-opioid receptor is believed to be mediated via common mechanisms with other opioids receptors agonists previously discussed in chapter 3.

The result of this experiment indicates that although the delta-opioid receptor, like other opioid receptors, is primarily distributed on lamina I-II of spinal dorsal horn (Besse et al., 1990; Maher et al., 2001) the effects of DPDPE are predominant on the pre-synaptic neurones, and the spinal analgesic effect of DPDPE on deep dorsal horn neurones was not altered by the depletion of NK1 expressing neurones in the superficial lamina. This study is in agreement with Nichols et al., (1999) study, where they used spinal infusion of morphine in SP-SAP treated animals and found no alteration in the degree of morphine analgesia and suggested that NK1 expressing neurones in the dorsal horn are not the major side of action of morphine. This finding may well be extended to others opioid receptor agonists such as this delta-opioid agonist. Although there is no direct data on the co-localization of opioid receptors and NK1 receptors on neurones of the superficial laminae the fact that the spinal analgesic effect of nociceptin was slightly attenuated in animals without lamina I NK1 expressing neurones further underlines the difference between ORL_1 receptor and other opioid receptors.

The results may suggest the co-localisation of NK1 and ORL_1 receptors and therefore by killing those neurones which present 10% of the neuronal population in lamina I (Brown et al., 1995; Mantyh et al., 1997) a slight attenuation of the nociceptin analgesia in deep dorsal horn neurones is produced. Another explanation could be the importance of the depleted neurones on supraspinal modulation of spinal transmission, since 80% of lamina I expressing NK1 neurones are projection neurones (Todd, 2002) and the lost of these neurones may cause disruption of descending excitatory or inhibitory pathways (Urban and Gebhart, 1999) that in turn could alter opioid function. Furthermore, it has been hypothesized that the loss of lamina I neurones expressing NK1 receptors projecting to the PB areas may turn to activate supraspinal site such as PAG and subsequently activation of RVM controls the spinal excitability through descending serotonin pathways. This spino-bulbo-spino-loop, proposed by Suzuki et al., (2002) may also explain the reduced analgesic response seen with SP-SAP animals after intrathecal injection of nociceptin and also the reduced heat coding response that was found in SP-SAP animals.

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CHAPTER 8

THE EFFECTS OF OXYTOCIN ON SPINAL NOCICEPTIVE PROCESSES

8.1 INTRODUCTION

Chronic pain after nerve or tissue injury as previously discussed in the introduction, includes a variety of symptoms and clinical characteristics; spontaneous and repetitive pain episodes, allodynia, hyperalgesia, and abnormal temporal summation of pain being the most frequent and representative symptoms. Increased knowledge about the pathophysiological mechanisms related to pain and analgesia may aid us to develop more effective therapies.

One important area is endogenous systems that may control pain. (Magoum, 1940) described a descending hypothalamic pathway to the spinal cord that may be involved in autonomic function. In fact, the hypothalamic paraventricular nucleus (NPV) reaches the spinal cord by a direct pathway (Kuypers and Maisky, 1975; Saper et al., 1976) and using immunohistochemical procedures it was demonstrated that this descending pathway uses amongst others, the peptide oxytocin (OT) (Buijs, 1978). Moreover, using in situ hybridization, dynorphin and oxytocin mRNAs are expressed in the 40% of PV neurones projecting to the spinal cord, and enkephalin mRNA is present in 20% of these neurones (Hallbeck et al., 2001).

It is interesting to note that oxytocin has been studied in different experimental pain models in order to obtain evidence for its analgesic effect (Arletti et al., 1993; Lundeberg et al., 1994; Ge et al., 2002; Robinson et al., 2002; Condes-Lara et al., 2003; Yu et al., 2003). Also, in humans lower oxytocin concentrations in spinal fluid and plasma were associated with pain states (Alfven et al., 1994; Yang, 1994), and oxytocin administration relieved the pain sensation (Madrazo et al., 1987; Yang, 1994).

On the other hand, several studies reported that systemic oxytocin did not produce any analgesic effect (Millan et al., 1984; Xu and Wiesenfeld-Hallin, 1994).

In an attempt to clarify the role of oxytocin in pain, I performed the present study to document the effect of different oxytocin doses on the responses of dorsal horn neurones to electrical and mechanical stimulation in normal and neuropathic rats.

8.2 RESULT

Electrophysiological recordings were made from normal (n=36) and neuropathic (n=12) rats. The mean depth of the recorded cells was 788 ± 23 μm, and there were no differences between normal and neuropathic rats in depths. The recorded cells showed evoked responses corresponding to the wide dynamic range category and based on their depth, were located in lamina V–VI. The normal rats were divided into groups of 6 and each group was used to test one dose, repeated twice, of the following oxytocin concentrations: 0.05, 0.1, 2, 5 and 10 International Units (I.U.). Each dose was followed for 1h and after that the drug was removed and the spinal cord was washed with saline and followed for 30 min before reapplying the same oxytocin dose. The same procedure was also applied for the neuropathic animals but here only doses of 1and 2 I.U. of oxytocin were used.

8.2.1 Effects of oxytocin on responses evoked by electrical stimulation in normal animals

The receptive fields of the recorded cells were found mainly on the toes of the ipsilateral hindlimb. Electrical stimulation of the receptive fields produced an activation of the recorded cells consisting of Aβ-, Aδ-, C-fibre and Post-discharge evoked responses (Fig.1) as well as input and wind-up. The analysis of these responses with respect to different doses of oxytocin did not show a linear dose response relationship. Thus, an oxytocin dose of 0.05 I.U. produces a facilitatory effect, while doses of 0.1, 1, 2, 5, and 10 I.U. produced a progressive decrease in the response. None of these responses was significant when compared with the control

values (these results are not illustrated). Two experiments in normal rats using 2 I. U. of oxytocin are shown (Fig.2 and 3), and these figures can be compared to those obtained in neuropathic rats (see below).

8.2.2 Effects of oxytocin on responses evoked by mechanical stimulation in normal animals

Application of von Frey forces produced progressive increases in the neuronal firing rate proportional to the forces applied. As with the electrical stimulation, the peptide effects on von Frey responses did not present a linear dose response relationship. In general, an oxytocin dose of 0.05 I.U. tended to increase the responses, while increasing oxytocin doses progressively decreased the responses. However, neither the increases nor the decreases were significantly different from control values.

8.2.3 Effects of oxytocin on responses evoked by electrical stimulation in neuropathic animals

An oxytocin dose of 1 I.U. produced a significant reduction in C-fibre ($P< 0.05$) and Post-discharge ($P< 0.05$) responses to electrical stimulation of the receptive field in neuropathic rats. Oxytocin dose of 2 I.U. produced a further pronounced diminution in the post-discharge, wind-up, and input values. The reduction of the post-discharge in neuropathic rats using 1 and 2 I.U. was the most marked change and was significant ($P< 0.05$) at 10 and 20 min after oxytocin administration (Fig.5 and 4). The post-discharge reduction was observed on the first 300–800 ms of the post-discharge (Fig.4B), but was also observed that the reduction in the post-discharge could be observed on the responses spanning the 2 s interval before the next stimulus in the train of 16 stimuli (Fig.5). Examples of the effect of 1 and 2 I.U. of oxytocin on the post-discharge are shown in Fig.4 and 5. In Fig.5 the post-discharge was analyzed

during the 300 to 1900 ms period following the stimulation and it shows a significant reduction ($P< 0.05$) after the peptide.

8.2.4 Effects of oxytocin on responses evoked by mechanical stimulation in neuropathic rats

As in normal rats, the von Frey stimulation with increasing filament force produced a progressive increase in the firing rates. Doses of 1 I.U. of oxytocin produced a progressive reduction in the activation of dorsal horn neurones evoked by the von Frey filaments in neuropathic animals. It is interesting to note that oxytocin significantly reduced ($P< 0.01$ and $P< 0.05$) the activation produced by the strongest and noxious mechanical stimulation (15, 30, and 75 g von Frey filament) see Fig.6B. Furthermore, oxytocin doses of 2 I.U. were also selective, affecting the highest von Frey stimulation forces (30 and 75 g).

8.2.5 Comparisons between neuropathic and normal rat responses

The effect of 1 and 2 I.U. of oxytocin was compared in both animal groups, both neuropathic and normal rats. The main effect of oxytocin was a significant reduction in the post-discharge evoked by electrical stimulation of the receptive field in the neuropathic rats, as seen by comparisons between Fig.2 vs. 4 and 3 vs. 5. Also, Fig.5 presents histograms showing the significant reduction ($P< 0.05$) at 10 and 20 min after application of 2 I.U. of oxytocin and a partial recovery at 30 min in neuropathic rats. On the contrary, in normal rats (Fig.3) no reduction in the post-discharge was observed. The post-discharge response comparison between normal and neuropathic rats using 1 and 2 I.U of oxytocin is displayed in Fig.7. Using 1 I.U of oxytocin, a significant ($P< 0.05$) reduction was observed only when a second dose of oxytocin was applied. Using 2 I.U. of oxytocin, the first application produced a significant reduction, a partial recovery after wash, and another significant reduction after the

second oxytocin dose. It is noteworthy that with 2 I.U. of oxytocin I observed an increase in the post-discharge in normal rats.

The responses to mechanical stimulation by von Frey filaments were also reduced more by oxytocin in neuropathic rats when compared with the control situation. This reduction was more pronounced and significant when von Frey filaments with higher forces are considered. These results are displayed in Fig.6. where clearly shows that the control rate frequencies appear higher but are not significantly different in neuropathic rats when they were compared with the normal rats.

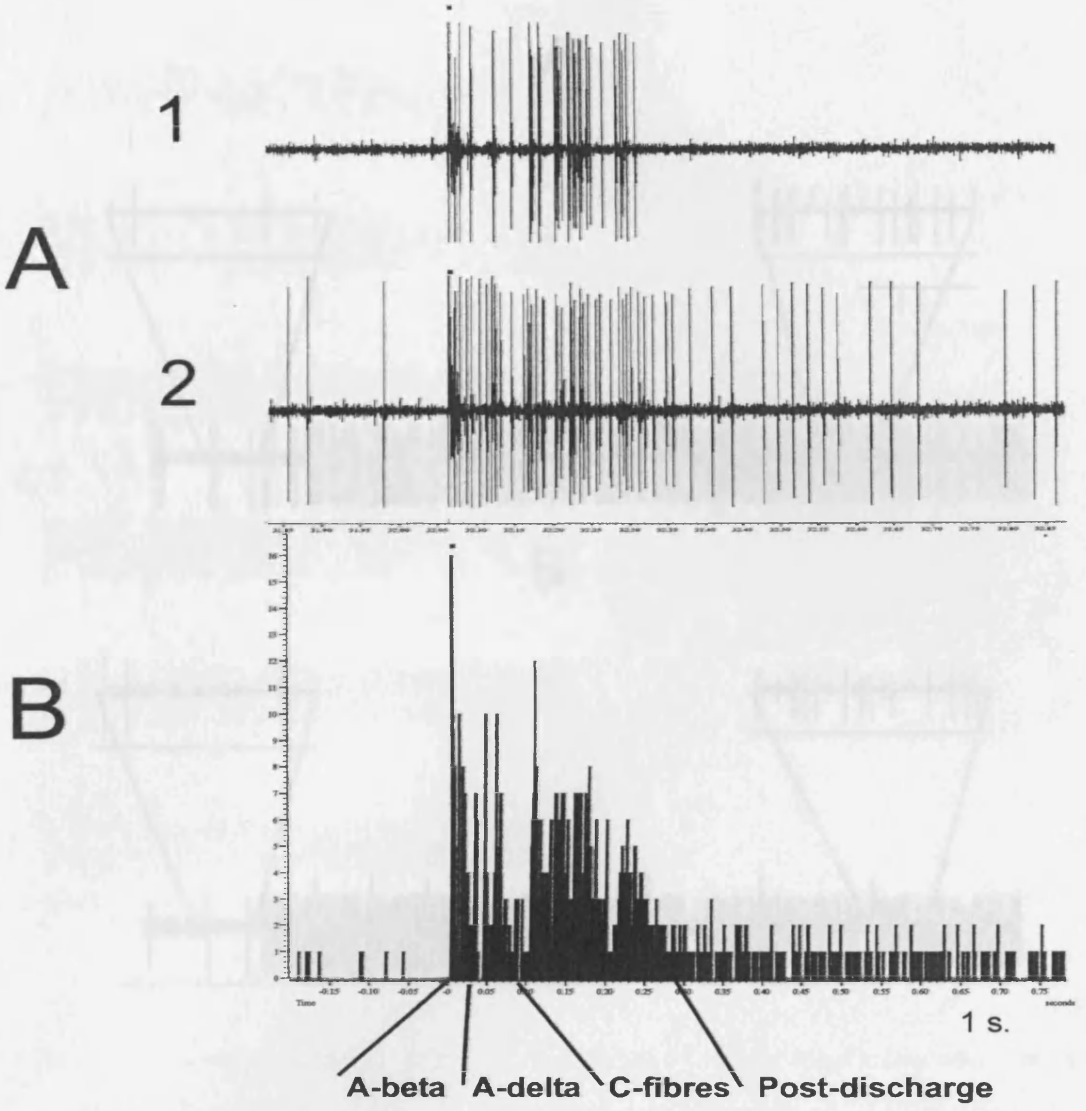

Figure 1. The response of single dorsal horn neurone on electrical stimulation. A1) The 2nd and A2) The 14th responses to a train of 16 stimulations are displayed; notice the increased response in C-fibre and post discharge evoked responses in A2. B) Shows a PSTH, in which can be observed 200 ms prior to the stimulation and the different fibres components of the response: Aβ-, Aδ-, C-fibre, and the post discharge.

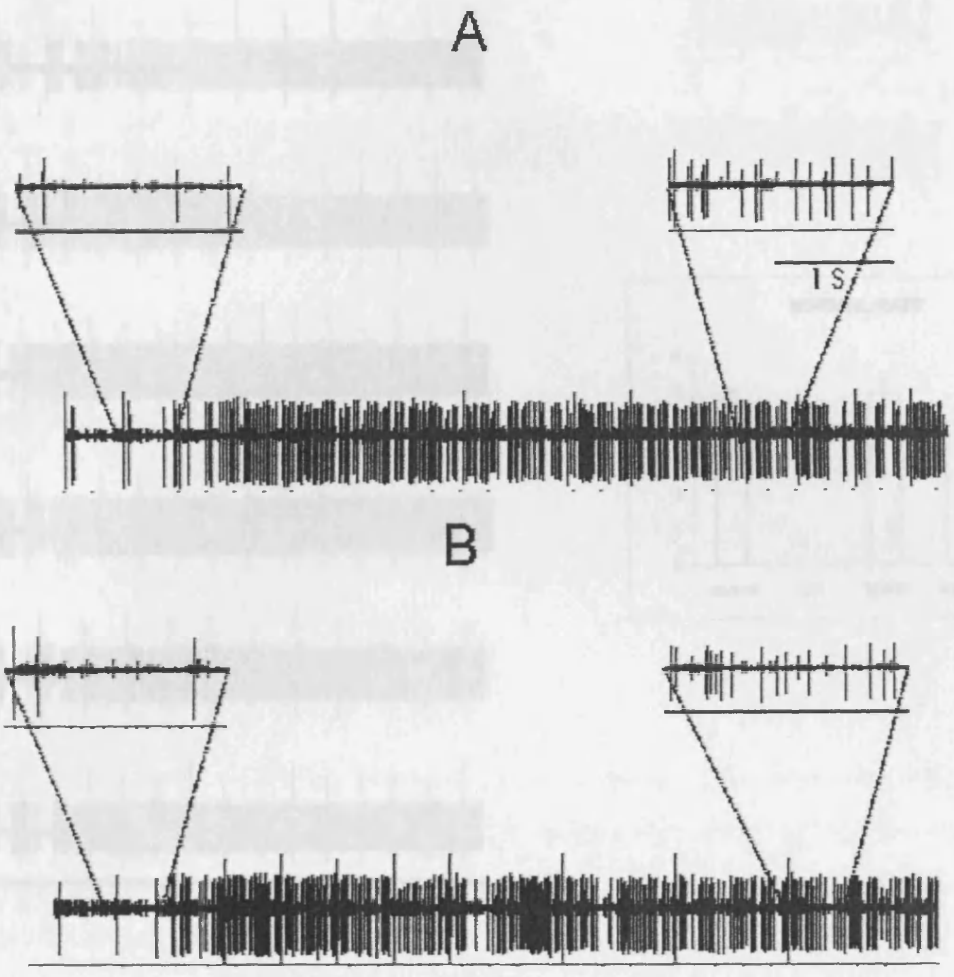

Figure 2. The figure shows the response of single dorsal horn neurone to the 16 electrical stimulations in: (A) control and (B) during intrathecal administration of oxytocin 2 I.U. in normal animal. In particular the window expands the enhancement of the post-discharge during the 2nd and the 13th stimulation that is not changed by the application of oxytocin (B) when compared to the 2nd and the 13th stimulation of control situation (A) respectively.

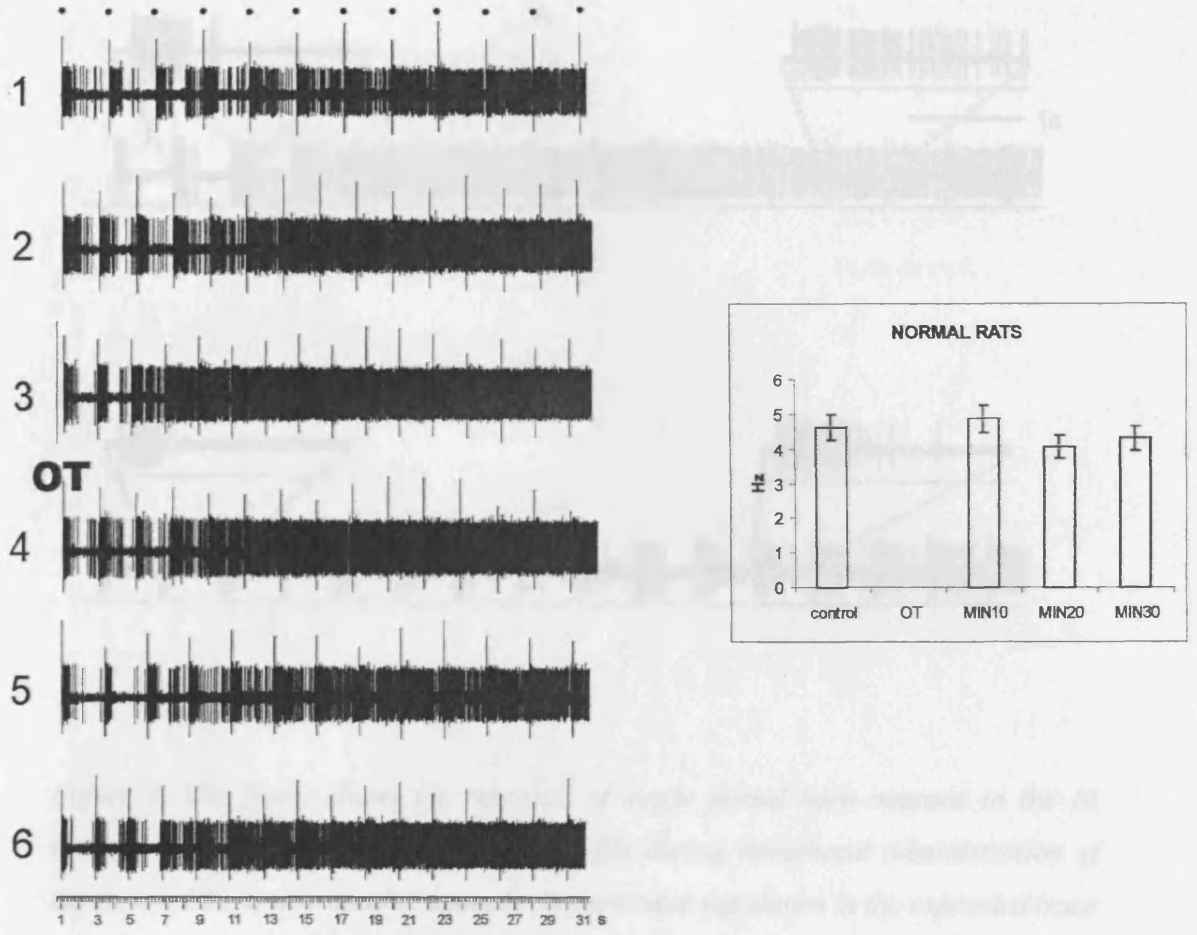

Figure 3. On the left side are plotted 6 recordings of 16 electrical stimulations in the control (1, 2 and 3) and during the intrathecal administration of 2 I.U. of oxytocin in a normal animal. In the upper part of trace 1, dots indicate the stimulus artifact. Notice that the post discharge in traces 4, 5, and 6 remains similar to the control. Each record has a 10 min interval. The histogram on the right shows the mean ± S.E.M. obtained between 300 to 1900 msec of the post-discharge. Oxytocin treatment caused no significant changes.

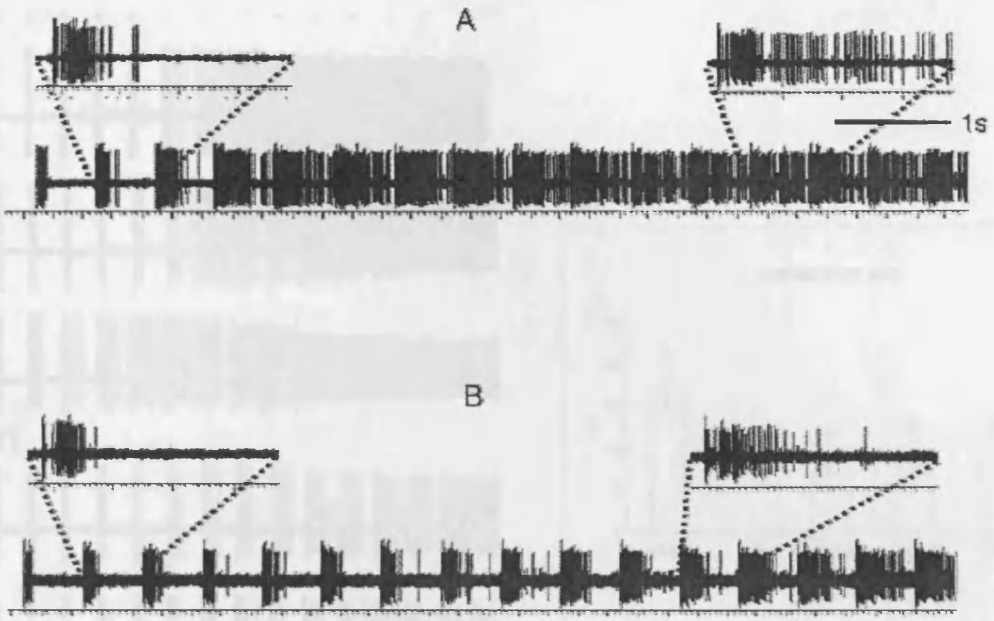

Figure 4. The figure shows the response of single dorsal horn neurone to the 16 electrical stimulations in: (A) control and (B) during intrathecal administration of oxytocin 2 I.U. in neuropathic animals. In particular (as shown in the expanded trace within the window), the enhancement of the post-discharge during the 2nd and the 13th stimulation shown in control situation (A) and clear reduction in this enhancement seen 10 min after the application of oxytocin (B).

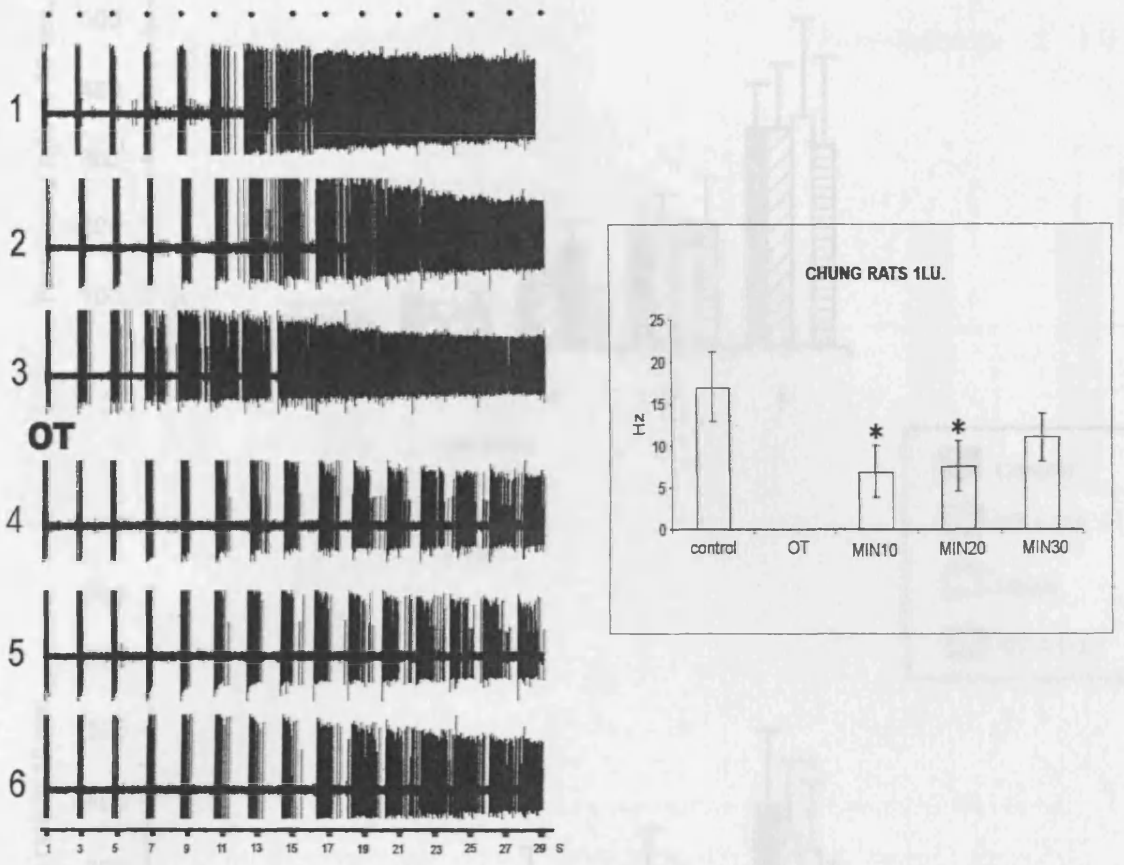

*Figure 5. This figure is similar to Fig.3, but it is during the intrathecal oxytocin administration of 1 I.U. in Chung (neuropathic) animals. In the upper part of trace 1, dots indicate the stimulus artifact. Notice the reduction in the post discharge in traces 4, 5, and 6. Each record shows a 10 min interval. The histogram at the right shows the mean ± S.E.M. obtained between 300 and 1900 msec of the post-discharge. Notice a significant (*P< 0.05, using Tukey and ANOVA tests) reduction at 10 and 20 min after 1 I.U. of intrathecal oxytocin.*

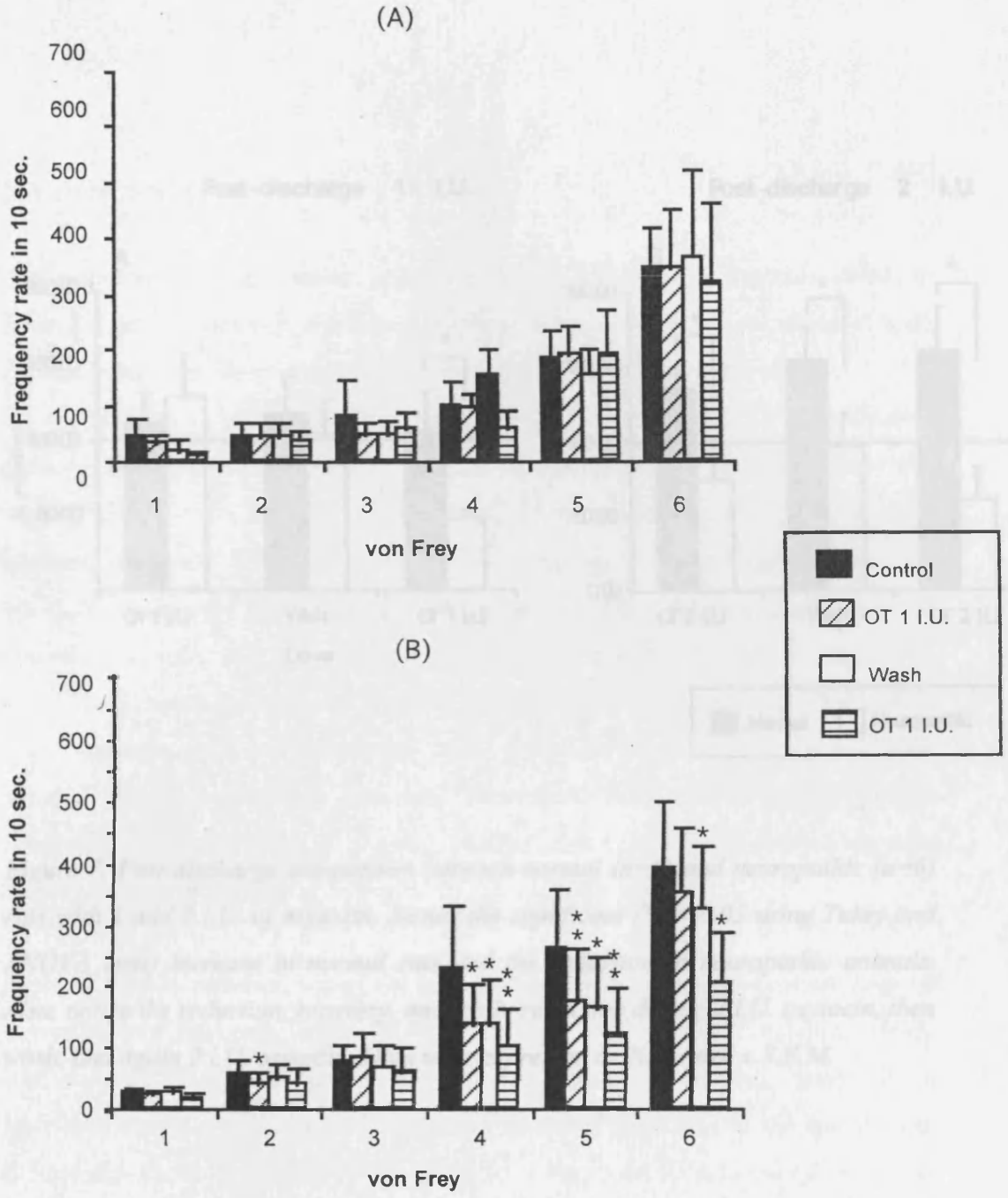

*Figure 6. Comparison of the neuronal responses to von Frey mechanical stimulation in (A) normal (n=6) and (B) neuropathic (n=6) rats. Normal rats did not show significant differences between control, oxytocin 1 I.U., wash, and oxytocin 1 I.U. (second dose). In contrast, significant differences (*P< 0.05, **P< 0.01 using Tukey and ANOVA tests) were found in neuropathic animals versus control values when used the von Frey filaments 4, 5, and 6. Data were expressed as mean number frequency rate (action potentials) evoked over 10 sec ± S.E.M.*

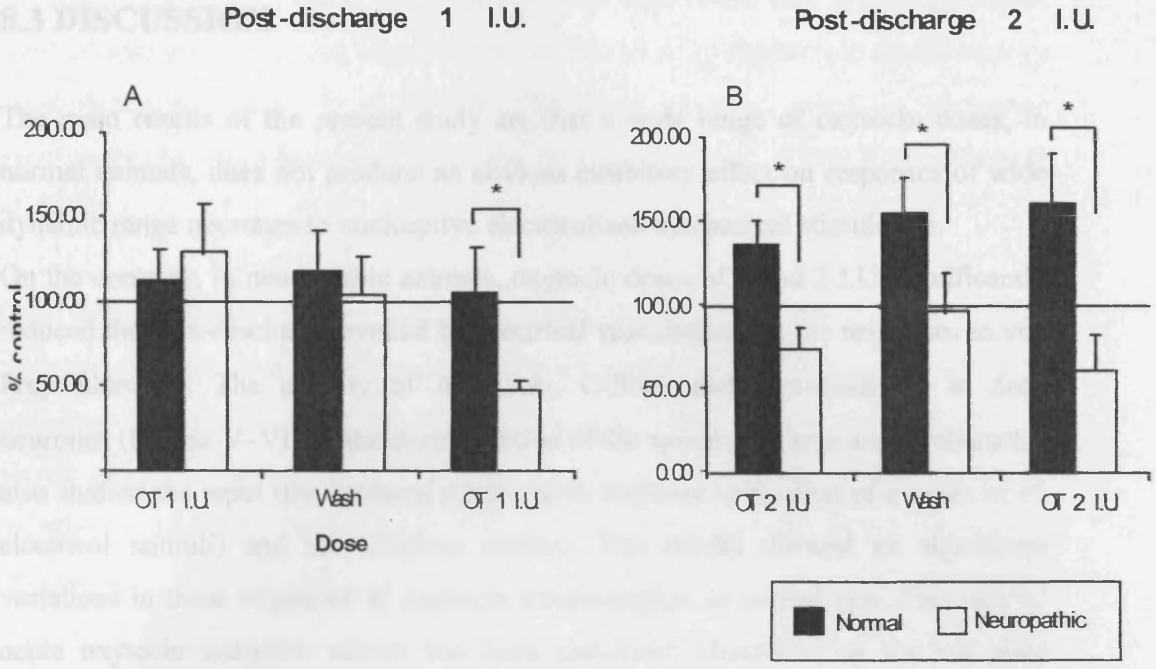

*Figure 7. Post-discharge comparison between normal (n=6) and neuropathic (n=6) rats with 1 and 2 I.U. of oxytocin. Notice the significant (*P< 0.05 using Tukey and ANOVA tests) increase in normal rats and the reduction in neuropathic animals. Also, notice the reduction, recovery, and again reduction during 2 I.U. oxytocin, then wash, and again 2 I.U. oxytocin. Data were expressed as % control ± S.E.M.*

8.3 DISCUSSION

The main results of the present study are that a wide range of oxytocin doses, in normal animals, does not produce an obvious inhibitory effect on responses of wide dynamic range neurones to nociceptive electrical and mechanical stimulation.
On the contrary, in neuropathic animals, oxytocin doses of 1 and 2 I.U. significantly reduced the post-discharge evoked by electrical stimulation and the responses to von Frey filaments. The activity of Aβ-, Aδ-, C-fibre, and post-discharge in deep neurones (lamina V–VI) of the dorsal portion of the spinal cord was also evaluated. I also studied the input (the neuronal discharge in response to the first of a series of 16 electrical stimuli) and the wind-up process. The results showed no significant variations in these responses to oxytocin administration in normal rats. This lack of acute oxytocin analgesic effects has been previously described on the hot plate latency test in normal rats (Xu and Wiesenfeld-Hallin, 1994) using systemic administration of low doses. In contrast, a higher dose of oxytocin increased hot plate latency, which may be due to non-specific effects of the peptide.

Motor blockade, sedation, vasoconstriction, or decrease in skin temperature may all confound any analgesic effect of oxytocin (Xu and Wiesenfeld-Hallin, 1994). Furthermore, lesions of the paraventricular hypothalamic nucleus, which is an important source of the oxytocin containing neurones projecting to the spinal cord, did not change the basal nociception threshold at the spinal level in the rat (Millan et al., 1984). Also, experiments done in oxytocin-knockout mice indicate that these animals show normal behavioral responses to noxious thermal and mechanical stimuli (Robinson et al., 2002). My findings in normal rats do not agree with some previous results (Condes-Lara et al., 2003) claiming an inhibitory or analgesic oxytocin effect (Arletti et al., 1993; Lundeberg et al., 1994; Yang, 1994; Ge et al., 2002; Yu et al., 2003).

Other studies suggest that the analgesic oxytocin effects are mediated, at least in part, by mu and kappa opioid receptors (Lundeberg et al., 1994; Ge et al., 2002; Lund et

al., 2002; Yu et al., 2003). Oxytocin analgesic effects are also related to stress-induced analgesia, in which oxytocin acts by inhibiting glutamatergic spinal sensory transmission (Robinson et al., 2002). This latter oxytocin action on glutamate mechanisms has been previously proposed elsewhere (Jo et al., 1998; Condes-Lara et al., 2003).

The use of different experimental pain models, routes of administration, and doses appear to determine whether this peptide has an analgesic effect. In the present study, important differences were obtained in neuropathic rats, never previously studied, showing a reduction of the post-discharge produced by electrical stimulation of the receptive field and also a significant reduction of the mechanical responses produced by von Frey filaments. Both post-discharge and mechanical responses are reduced more by 2 I. U. than by 1 I. U. of oxytocin. The different results obtained in normal and neuropathic animals revealed an important distinction between these animals, illustrating plastic changes occurring as a consequence of nerve injury.

In normal animals one of the proposed mechanisms of oxytocin is that, by acting on the oxytocin receptor, a G protein-coupled receptor (Gq), oxytocin mobilizes intracellular Ca^{2+} and opens a non-specific cationic channel or closes a K^{+} channel (Gimpl and Fahrenholz, 2001). In neuropathic animals there is an up regulation of the alpha 2 delta subunit of calcium channels after nerve injury and increases of both N type (nerve injury) and P type (tissue damage) calcium channels after pathological changes (Dickenson et al., 2002), which might explain the inhibition obtained with oxytocin in neuropathic animals.

The ability of oxytocin to reduce consequences of N-methyl D- aspartate receptor events such as wind-up and post-discharges. Relates to findings that (Condes-Lara et al., 2003), oxytocin could block glutamate activation and reduce nociceptive evoked responses in spinal cord cells. NMDA receptors are implicated in wind-up (Dickenson and Sullivan, 1987; Willis et al., 1996), and intrathecal injection of oxytocin produces hyperalgesia (Kitto et al., 1992). In addition NMDA receptor

antagonists produce a marked, dose-dependent inhibition of C-fibre evoked responses (Haley et al., 1992).

Other possible explanation of my findings would include changes in the brainstem and midbrain that occur after peripheral nerve damage. Ascending noxious information could project to areas such as the hypothalamus paraventricular nucleus, which projects to the spinal cord and is an important source of oxytocin. Both ascending and descending activity could change after nerve injury and may alter oxytocin levels and receptor function at the spinal cord. This could be a possible explanation of our findings. Further studies on the oxytocin peptide may reveal important links between endocrine peptides functioning as neurotransmitters and altered pain states.

Finally, our study suggests that oxytocin has potential therapeutic value as an endogenous antinociceptive agent, a target that could lead to drug development.

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CHAPTER 9

FINAL DISCUSSION

The spinal cord is an important part in the functional organization of the central nervous system. In particular it has a vital role in the integration, modulation and control of sensory information as well as transmitting this information to higher brain centres. This has made the spinal dorsal horn a target for many pain studies. The studies used in my thesis have been developed for the purpose of analyzing in vivo mechanisms of pain. This type of study has the advantage of directly activating the primary afferent by electrical stimulation and/or by natural stimuli such as mechanical and heat stimuli. This allows the study of the effects of a wide range of noxious and innocuous stimuli of different natures. Importantly, the direct measures of neuronal activity, involved in the sensory aspects of pain, allows observation of supra-threshold measures, not possible in behavioural studies where the endpoint is a threshold withdrawal. This allows measures closer to the clinical issues of pain in patients where pain levels are above the threshold. Furthermore, the model should not be complicated by secondary effects of drugs such as sedation or motor effects that could confound behavioural measures. Moreover, direct application of the drug into the spinal cord will permit the study of the pharmacological effect of particular compounds within a complex neuronal network, which reproduces the physiological environment where the drug will be acting. However, it must be said that the model only studies efficacy and here drugs may inhibit neuronal responses at doses that cause unacceptable side-effects.

In addition the dorsal horn of the spinal cord is subject to a great deal of plasticity following pathophysiological changes such as inflammatory and neuropathic related pain which presents a clinical problem. Therefore understanding this dynamic circuit will enable better management of these painful states.

In this thesis the pharmacological effects of several compounds of interest were investigated on deep spinal dorsal horn neurones (V-VI) which are primarily WDR neurones, and this was done in different models of pain states.

One of the major compounds investigated here was nociceptin, the endogenous ligand of the fourth opioid receptor, the ORL_1 receptor. In this study it was confirmed that intrathecal application of nociceptin induces analgesic effects on spinal dorsal neuronal responses of deep laminae dorsal horn cells in normal rats. This effect was a dose-dependent one as has been previously reported by a study done in our laboratory (Stanfa et al., 1996) and in others (Xu et al., 1999). The lack of effect of nociceptin on the Aβ-fibre responses and the greater inhibition seen on the C-fibre evoked responses suggests a predominant effect of this compound at pre-synaptic sites (action either on afferent terminals or neuronal systems pre-synaptic or upstream to the recorded neurone) as direct post-synaptic actions appear less likely due to this selective effect of nociceptin on C-fibre responses. However, in accord with Stanfa et al., (1996), I found that nociceptin also had a potential action through post-synaptic opioid receptors on neurones and hence was able to inhibit post-synaptic mechanisms such as wind-up and post-discharge of the recorded neurones. These potential pre-and post-synaptic actions of nociceptin are supported by the proposed mechanisms of action of nociceptin; for instance nociceptin inhibits N-type calcium channels and it is also believed that nociceptin causes membrane hyperpolarization by increasing K^+ conductance and inhibits adenylate cyclase (Connor et al., 1996; Meunier, 1997). Overall these effects attenuate the propagation of action potentials and release of neurotransmitters in the afferent sensory nerve fibres in a similar way to that proposed by the mechanisms of action of the other opioids (Dickenson, 1994; Sawynok, 2003).

Using the top dose of nociceptin in neuropathic animals where the spinal nerves L5 and L6 were tightly ligated I observed a similar analgesic effect, but this effect was less when compared with the normal animals. Although several behavioural and *in vitro* studies suggest the effectiveness of nociceptin in other neuropathic models (Yamamoto and Nozaki-Taguchi, 1997; Yamamoto et al., 1997; Abdulla and Smith, 1998), there is limited information about the basis for the observed electrophysiological changes in the nociceptin system in the spinal cord following nerve injury and chronic inflammation. I have therefore seen that the potency of

nociceptin was higher in normal animals followed by animals with nerve injury and sham operated animals were the least effected which futher emphasizes the changes seen after nerve ligation since these animals received the same preliminary surgery as the sham animals. Similar discrepant effects of nociceptin were seen in sham operated animals when compared with carrageenan animals in the (Carpenter et al., 2000) study. Even though both animals exhibit a degree of peripheral inflammation one main difference is that the sham animals were used at 2 weeks whereas previous studies looked at acute effects of inflammation suggesting that the regulation of ORL_1 receptors may differ with time.

The mechanisms behind the reduced effect of nociceptin in neuropathic animals and the lack of effect in animals with chronic inflammation need further investigation. However it is possible that both pain states may present plasticity in peripheral and central nervous systems that could further enhance the response of the dorsal horn neurones more than in normal states so that inhibition is less as a result of higher levels of excitation and subsequently nociceptin may be less effective in those animals. Moreover these findings indicate that peripheral inflammation and nerve injury may regulate endogenous nociceptin and its receptor in ways different from or opposite to the classical opioid system.

To clarify the role of this peptide nociceptin and its receptor much research has been directed towards the discovery of non-peptide agonists and antagonists at the ORL_1 receptor. A novel ORL_1 receptor ligand, named by Solvay Pharmaceuticals as compound 20700857 was for the first time investigated here on deep spinal dorsal horn neurones. The compound in study did not produce the spinal antinociceptive effect seen with nociceptin, suggesting that compound 20700857 may have less potency or specificity or may not possible act on ORL_1 receptor. However the complex effect on wind-up evoked response which includes both facilitatory and inhibitory effects may indicate a role of this compound on mechanisms behind neuronal excitability.

The discovery of a selective nociceptin antagonist J-113397 by Kawamoto et al., (1999) and Ozaki et al., (2000) made possible the further investigation of the nociceptin system. Using J-113397 after obtaining the maximum inhibition with cumulative doses of nociceptin, I clearly demonstrated almost a complete reversal by this compound of the effects of nociceptin on spinal dorsal horn neurones in normal animals. This should prove to be a useful tool for the study of this receptor system. This data is in agreement with the studies indicating that J-113397 blocks the ORL_1 receptor in a competitive manner and possesses high affinity and selectivity for the ORL_1 receptor (Ozaki et al., 2000; Ichikawa et al., 2001). To further provide evidence of the selectivity of J-113397 on ORL_1 over the mu-opioid receptor i.t. administration of 1 μg of morphine after the administration of J-113397 still induced the marked inhibitory effect expected by morphine. J-113397 did not produce any antagonistic effect on intrathecal oxycodone, another opioid agonist thought to be acting through the mu- and/or kappa-opioid receptor (Beaver et al., 1978; Yoburn et al., 1995; Ross and Smith, 1997). These findings demonstrate that J-113397 has no interaction with the other opioid receptors. Moreover, through this study I found that the spinal analgesic effect of oxycodone was less than the effect of morphine obtained in similar studies (Suzuki et al., 1999) and higher than spinal analgesic effect of nociceptin.

Using this selective antagonist of nociceptin actions at the ORL_1 receptor, it was possible to elucidate any potential endogenous activity of nociceptin at the ORL_1 receptor in different pain models. It was found that J-113397 had no significant effect on the electrically or naturally evoked recordings made from deep dorsal horn neurones in normal animals and this was not altered after inflammation and neuropathy. Overall, the data indicated that endogenous nociceptin has no or only a minor role in spinal nociceptive processing in those pain states. These findings correlate well with studies done in nociceptin receptor-knockout mice, where it was shown that basal nociceptive thresholds were unchanged in these animals (Nishi et al., 1997; Mamiya et al., 1998). The use of this antagonist may help explain the functional roles of nociceptin and its receptor in the brain and spinal cord, for review see (Calo et al., 2000; Mogil and Pasternak, 2001).

The effect of opioids in neuropathic pain is a matter of considerable controversy (chapter 3). The increases in spinal CCK during neuropathic pain and subsequent reduction in the potency of spinal morphine led to suggestions of a role of this peptide in this pain state (Xu et al., 1993). Additionally, CCK antagonists were seen to potentiate morphine analgesia (Watkins et al., 1985; Stanfa et al., 1994).

Since nociceptin acts at ORL_1 which is an opioid-like receptor, it was of interest to investigate whether CCK has an effect on the nociceptin/ORL_1 system. Their interaction was studied in this thesis for the first time on the spinal cord neuronal responses in normal, neuropathic and sham operated rats. In normal animals, pretreatment with CCK prevented the inhibitory effect of nociceptin and therefore CCK had the expected 'anti-opioid' action while in neuropathic and sham operated animals, CCK enhanced the analgesic action of nociceptin. The enhanced effect of nociceptin obtained after treatment with CCK in sham operated animals could be as a result of surgical inflammation. This was also found with morphine (Stanfa and Dickenson, 1993) and this enhancement has been attributed to decreases in the availability of endogenous CCK (Stanfa and Dickenson, 1993). As a consequence there are potential similarities between spinal mu-opioid and ORL_1 receptor function in normal animals and after inflammation. However, in nerve injury, CCK not only failed to block the effects of nociceptin but enhanced the inhibitory effects of the peptide. This synergism suggests a plasticity in the actions of CCK/nociceptin and further emphasises that the ORL_1 receptor has very different characteristics from the mu-opioid receptor at spinal levels.

Since CCK interacts with intracellular mechanism of calcium regulation (Wang et al., 1992) and in neuropathic pain there is an upregulation of the alpha 2 delta subunit of calcium channels and enhanced actions of both N (nerve injury)- and P (tissue damage) -type calcium channels after pathological changes (Dickenson et al., 2002), suggesting that the CCK/ORL_1 interaction may involve intracellular calcium. Another possible mechanism is that CCK, acting on CCK_B receptors, decreases the

availability of the enkephalins (Nichols et al., 1996). From these findings it appears that the ORL_1 receptor may be a useful target for the treatment of nerve injury and other pain states.

The effect of nociceptin was also examined in animals where lamina I NK1 expressing neurones have been ablated using a saporin-substance P conjugate (SP-SAP) and was compared to a control group (saline or SAP). In this study it was shown that in NK1 neuronal ablated animals the response of intrathecal nociceptin on deep dorsal horn neurones was attenuated when compared with the control group. Although the greatest change was seen on the wind-up response, which is an intrinsic spinal phenomenon relying on circuit between lamina I-lamina V (Dickenson et al., 2004), overall the neuronal response was slightly changed due to the lack of lamina I NK1 expressing neurones. To further analyze the importance of these neurones on the responses of deep dorsal horn to opioids, I also assessed the effect of a delta-opioid receptor agonist, DPDPE, in saline, SAP and SP-SAP animals. It was found that the spinal analgesic effect of DPDPE on deep dorsal horn neurones was not altered by the depletion of NK1 expressing neurones in the superficial lamina. This was in accord with (Nichols et al., 1999) study, suggesting that the NK1 expressing neurones in the dorsal horn are not the major site of action of morphine which is not surprising given the large proportion of pre-synaptic neurones. Thus this finding may well be extended to other opioid receptor agonists such as delta-opioid agonist. The attenuation of the spinal analgesic actions of nociceptin by this treatment further underlines the difference between ORL_1 receptor and other opioid receptors and this may suggest that post-synaptic ORL_1 receptors have greater contributions to the effect of nociceptin when compared to other opioid receptors. Another explanation could be that NK1 and ORL_1 receptors may be co-localised on the same neurones and by ablating these neurones which present 10% of the neuronal population in lamina I (Brown et al., 1995; Mantyh et al., 1997) may reflect the slight attenuation of the nociceptin analgesia in deep dorsal horn neurones.

Moreover, since the majority of lamina I expressing NK1 neurones project to the brainstem areas implicated in the affective and discriminative aspect of pain (Mantyh

et al., 1997; Todd et al., 2000). The disruption of these pathways may activate the descending suparspinal mechanisms and as proposed by Suzuki et al., (2002) the spino-bulbo-spino-loop descending facilitation may well explain the reduced analgesic response seen with SP-SAP animals after intrathecal injection of nociceptin and also the reduced heat coding response that was found in SP-SAP animals.

The final pharmacological study presented here is the spinal effect of the neurohormone, oxytocin. The spinal effect of oxytocin in normal animals is controversial. Here I found that spinal application of oxytocin did not produce any significant change in the electrical and mechanical evoked response in normal animals, while in neuropathic animals oxytocin produced inhibition of both responses. There are several suggestions for these results, for instance since one of the proposed mechanism of oxytocin involves intracellular calcium (Gimpl and Fahrenholz, 2001) and in neuropathic pain as previously seen with CCK there is upregulation of the alpha 2 delta subunit of calcium channels and enhanced actions of both N (nerve injury) and P (tissue damage) calcium channels, this may explain the inhibition obtained with oxytocin in this pain state. One other possibility could be that after nerve injury there might be changes at supraspinal levels that could alter the function of oxytocin system at the spinal cord. Indeed, further study of oxytocin at supraspinal levels will be required to fully elucidate the role of oxytocin in neuropathic animals.

Overall this thesis provides a better understanding of both exogenous and endogenous nociceptin on spinal dorsal horn neurones in different pain states. It was successfully shown that interaction between nociceptin and CCK enhanced the analgesic affect of nociceptin and thus the ORL_1 receptor may be useful target for the treatment of nerve injury and in chronic pain. This study further underlines the difference between the ORL_1 receptor with other opioid receptors and raised the possible involvement of population of lamina I neurones in the modulation of nociceptin response on deep dorsal horn neurones. In addition, this thesis emphasizes the plasticity of oxytocin system in neuropathic animals and its effectiveness in this pain state.

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Cholecystokinin fails to block the spinal inhibitory effects of nociceptin in sham operated and neuropathic rats

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Research report

Oxytocin actions on afferent evoked spinal cord neuronal activities in neuropathic but not in normal rats

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